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Studies of influenza virus tropism

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Studies of influenza virus tropism

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Sciences
Microbiology and Molecular Genetics
2017

Abstract

Studies of influenza virus tropism
By Hannah M. Creager

Influenza A viruses are capable of causing a wide spectrum of disease across a variety of avian and mammalian hosts. Pathogenicity and transmissibility are thought to be influenced by the cellular and tissue tropism of the virus. Animal models allow for study of tropism in the context of complex hosts, but imperfectly replicate human physiology. In contrast, in vitro studies allow for the examination of human tissue, and the isolation of specific cell types, but are typically of limited complexity. This work bridges the gap between these disparate types of studies by adding additional nuances to *in vitro* models. For example, infection of adherent cell monolayers using a liquid inoculum represents an established method to reliably and quantitatively study virus infection, but poorly recapitulates the exposure and infection of cells in the respiratory tract that occurs during infection with aerosolized pathogens. We therefore began by developing methodology to expose adherent mammalian cell monolayers to defined quantities of aerosolized influenza virus. We found that certain viruses were able to replicate productively in human primary alveolar epithelial cells following liquid, but not aerosol inoculation. Additionally, aerosolized virus was able to infect a human bronchial epithelial cell line in spite of a thick apical mucus layer resulting from three weeks of culture at air-liquid interface, but viral infectivity was reduced up to 25-fold. By facilitating study of viral infectivity under conditions similar to those of natural infection and transmission, this novel method has the potential to enhance our understanding of influenza and respiratory viruses.

Next, we applied aerosol inoculation to the study of ocular tropism. Though influenza viruses typically cause respiratory tract disease, some viruses, particularly those with an H7 hemagglutinin, have been isolated from the eyes of conjunctivitis cases. We asked whether conjunctivitis-associated viruses were unique in the ability to infect ocular cells by the aerosol route or to replicate efficiently at 33°C and found that they were not. Similarly, the membrane associated mucins expressed on differentiated corneal tissue constructs did not restrict the infection or replication of respiratory isolates. However, human tears significantly inhibited hemagglutination of both ocular and non-ocular isolates but the effect on viral infectivity was more variable, with tears reducing the infectivity of non-ocular isolates more than ocular isolates. These data suggest that most influenza viruses may be capable of establishing infection if they reach the ocular surface, but that this is more likely for ocular isolates as they are better able to maintain their infectivity in the presence of tears.

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Chapter 1: Introduction

There are four distinct genera of influenza viruses, all of which are members of the Orthomyxoviridae family: influenza A, B, C, and D (1). Influenza A viruses, which are the focus of this dissertation, are further classified into subtypes based on their surface glycoproteins. There are 18 subtypes of the viral hemagglutinin (HA) protein, which binds to sialic acid present on the surface of host cells and 11 subtypes of the neuraminidase (NA), which cleaves sialic acid from glycans. With the exception of HA types 17 and 18, and NA types 10 and 11, which have been detected exclusively in bats, all HA and NA subtypes circulate in aquatic birds, the natural reservoir for influenza A viruses (2-6).

Virus Structure and Replication

Influenza A viruses are enveloped RNA viruses with a negative sense genome consisting of 8 segments from which 10 major proteins plus several accessory proteins (not present in all viruses) are expressed (7). Virions are spherical or filamentous, and pleomorphic in shape; structure is provided by the viral matrix (M1) protein, which underlies and supports the viral envelope (8, 9). The HA and NA, as well as the M2 protein are embedded within the viral membrane. The trimeric HA serves as a receptor binding and fusion protein. Each monomer is initially produced as a single polypeptide called HA_0, which must be cleaved by host enzymes into HA_1 and HA_2 subunits to activate the protein's fusion potential (10, 11). Also present in the viral envelope is the viral M2 proton channel. Each of the eight gene segments wraps around oligomerized nucleoprotein (NP), which binds to a single heterotrimeric complex of the polymerase

proteins, forming a ribonucleoprotein (RNP) (12-15). The RNPs are arranged in a 7+1 pattern, with seven RNPs forming a ring around a single central RNP (16).

Infection begins with virion attachment to sialic acid present on the surface of host cells. The virion is then endocytosed, and as the endosome acidifies, protons enter the virion through the M2 channel (17, 18). This precipitates the dissociation of the ribonucleoproteins from the M1 protein (19). Decreasing pH of the endosome triggers a conformation change in the HA, which exposes the viral fusion peptide. This highly hydrophobic peptide inserts itself into host cell membrane, and an additional conformation change brings the viral and cellular membranes into close proximity, facilitating fusion, which releases the RNPs into the cytosol of the cell (20). Karyopherin α then ferries the RNPs to the nucleus, where transcription of viral mRNAs and replication of the genome occur (21). As new genomic RNA segments are produced, they are coated with NP, which associates with the viral polymerase complex. Binding of the RNP to the M1 and NEP/NS2 proteins allows it to be exported from the nucleus by Crm1 (22-27).

After reaching the cytoplasm, RNPs associate with a microtubule organizing center and are transported from there to the plasma membrane by $Rab11^{+}$ recycling endosomes (28-30). Here, they assemble with viral proteins to form nascent virions, which bud from the cell membrane. The exact steps involved in this process remain undefined, but it is known that packaging signals on each of the viral gene segments facilitate the incorporation of eight unique segments into each virion (7, 31). After budding, the viral neuraminidase removes sialic acid receptors from the cell and/or viral

membrane such that the new virions are able to diffuse away from the cell rather than remaining bound to sialic acid residues on its surface (32, 33).

Avian influenza

Wild aquatic birds are considered the natural reservoir of influenza A viruses. Viral genetic diversity is high within this population, with transmission occurring via the fecal-oral route. Infection is typically asymptomatic. Other avian species, including shorebirds, passerines, and domestic poultry are susceptible to infection as well. Avian viruses are classified as either low pathogenicity avian influenza (LPAI) or highly pathogenic avian influenza (HPAI) viruses depending on their ability to cause disease and death in chickens inoculated intravenously (34). HPAI viruses have been limited to the H5 and H7 subtypes, although it is important to note that the majority of H5 and H7 viruses are of low pathogenicity (35).

Poultry infected with LPAI may not appear ill, or may show mild to moderate clinical signs including respiratory disease, reduced consumption of food and water, and decreased egg production. Infection is limited to the respiratory, intestinal, and reproductive tracts as the trypsin-like serine proteases required for cleavage of the viral hemagglutinin are not present in other tissues (35). In contrast, HPAI (formerly known as fowl plague) viruses cause severe disease in gallinaceous poultry, particularly chickens. Because these viruses, with rare exceptions, have multiple basic residues at their HA cleavage site, they are cleaved by ubiquitous furin-like proteases and can therefore cause systemic infection (36, 37). Although infection typically begins in the nasal epithelium, HPAI viruses spread rapidly to the cardiovascular and lymphatic symptoms and disseminate throughout the body. Morbidity and mortality may result from circulatory

collapse triggered by viral replication in endothelial cells or from multi-organ failure (38).

Influenza in humans

Influenza infection in people is typically caused by viruses adapted to circulation within the human population. Historically limited to the H1N1, H2N2, and H3N2 subtypes, these are often referred to as seasonal influenza viruses because in temperate climates, outbreaks tend to occur on an annual basis during the winter. In tropical regions, infections occur throughout the year (39). Transmission can occur via several routes, the relative importance of which is a subject of ongoing debate: direct and indirect contact, fomites, large respiratory droplets, and aerosols (40-47). Influenza illness is typically characterized by both respiratory and systemic symptoms, including sneezing and coughing, fever, malaise, and myalgia. Disease onset is usually rapid, occurring one to four days after exposure (48). In uncomplicated influenza, viral replication remains confined to the upper respiratory tract and disease is self-limiting, lasting 3-7 days, although some symptoms such as malaise and cough may persist for a longer period. Young children, the elderly, pregnant women, and individuals with certain chronic medical conditions are particularly at risk for severe disease and complications, which include bacterial pneumonia, myocarditis, and exacerbations of chronic diseases such as asthma or diabetes (49). As a result, influenza is estimated to cause over 23,000 deaths annually in the United States, nearly 90% of which occur in persons ages 65 or older (50).

Pandemic influenza

Influenza pandemics occur when an antigenically novel virus evolves the ability to transmit efficiently between people. Unchecked by pre-existing population immunity, such a virus can cause widespread illness. Although historical evidence indicates that pandemic influenza has plagued humanity for at least several centuries, and possibly much longer, most scientific study has focused on the pandemics of the 20th and 21st centuries because viral isolates are not available from earlier time periods (51). The pandemics of 1957, 1968, and 2009 were all driven by reassortment, a process in which two distinct viruses infecting the same cell exchange gene segments, yielding progeny virions with some genes from each parent. The 1957 H2N2 pandemic virus resulted from a reassortment event or events which introduced avian HA, NA, and PB1 gene segments into the H1N1 virus which had been circulating previously. The HA and PB1 segments were again replaced by those from an avian source in the 1968 H3N2 pandemic virus (52, 53). A more complex series of reassortment events resulted in the 2009 H1N1 pandemic virus: first, Eurasian swine viruses acquired NA and M segments from avian viruses. A classical North American swine virus then reassorted with both human and avian viruses. Finally, the Eurasian and North American swine lineages reassorted, yielding the pandemic virus (54).

The origins of the virus responsible for the great 1918 influenza pandemic are less clear because no sequence data is available from viruses which predate the pandemic. This pandemic was notable for its severity: approximately one third of the human population became ill and an estimated 50 million or more, many of them young adults, died (55, 56). Secondary bacterial pneumonia is thought to be the cause of the most of the deaths, but 10-15% of cases were characterized by dramatic and rapid progression to

heliotrope cyanosis and death by pulmonary hemorrhage or resulting from the virus itself and the immune system's attempts to control it (56-59). Taubenberger *et al.* initially proposed that the 1918 virus moved directly from birds into humans, and Taubenberger subsequently clarified that this species jump likely occurred at least several years prior to 1918, allowing for human adaptation prior to the pandemic (60, 61). More recent studies indicate that while non-HA gene segments appear avian-like and diverged from the avian lineage in or later than 1903, one or more reassortment events between viruses circulating in humans, and possibly swine as well, were involved in generation of the pandemic virus (62-64).

Human infections with avian influenza

On occasion, avian viruses can be transmitted directly to humans, following direct or indirect contact with infected poultry. To date, human infections with five different hemagglutinin subtypes (H5, H6, H7, H9, and H10) and seven different NA subtypes (N1, N2, N3, N6, N7, N8, and N9) have been documented (65-73). Although none of these viruses has yet demonstrated an ability to sustain transmission from person-to-person (small clusters of probable transmission between humans have been identified), their antigenic novelty gives them the potential to cause a pandemic should they evolve this capability (74-84). Of particular concern are HPAI H5N1 and LPAI H7N9 and H9N2 viruses as these have caused the largest numbers of cases. Serologic evidence suggests that asymptomatic, or clinically insignificant infection occurs with each of these subtypes (85-89).

Among symptomatic cases, typical disease severity varies by subtype. H9N2 viruses, for example, tend to result in relatively mild illness, similar that that associated with human viruses (90). Infections with non-N9 H7 viruses, both LPAI and HPAI often cause conjunctivitis, sometimes accompanied by respiratory symptoms (91). In contrast, HPAI H5N1 viruses usually cause severe disease. Because these viruses replicate well in alveolar epithelial cells, the majority of cases develop a fulminant primary viral pneumonia. This can progress to acute respiratory distress syndrome, in which extensive damage to the epithelial-endothelial barrier of the lung allows fluid to flood the alveoli, interfering with gas exchange and leading to respiratory failure (92). While this is the most frequent cause of death, which occurs in approximately 53% of cases, disease is not limited to the respiratory tract (93). High levels of proinflammatory cytokines are present in the plasma, and leukopenia, lymphopenia, and thrombocytopenia are common hematological findings. Virus has been detected in the blood, feces, CSF, and viscera of several patients (94). However, the frequency with which virus spreads beyond the respiratory tract remains unclear due to the limited number of autopsies performed on those from whom infection has been fatal (95).

In March of 2013, the Chinese Center for Disease Control and Prevention reported the first human infections with an H7N9 virus. Since then, five annual epidemics have resulted in over 1500 confirmed cases (93). Although nearly all H7N9 isolates to date have been LPAI viruses, they have proven capable of causing disease which resembles that observed with HPAI H5N1 infection: fever and cough progress to viral pneumonia, which leads to acute respiratory distress syndrome or shock. There is no evidence of viral spread beyond the respiratory and gastrointestinal tracts, but disease is

nonetheless systemic, with many patients experiencing lymphocytopenia and thrombocytopenia, and high plasma levels of inflammatory cytokines (96). Differences in illness severity between LPAI infections and the eight HPAI H7N9 cases reported to date, all of which occurred during the fifth epidemic wave, have not been detected, but this may change as a result of increased statistical power if more HPAI H7N9 infections occur (97).

Despite the similarities in disease associated with HPAI H5N1 and LPAI H7N9 viruses, they affect different groups of people. Most H5N1 infections occur in previously healthy children or young adults (median age, 18 years) whereas H7N9 patients tend to be older (median age, 58 years) and are more likely to be male (70%) and to have underlying medical conditions (38%) (98). The majority of H5N1 cases report handling sick or dead poultry, usually those they raise themselves. In contrast, most H7N9 infections appear to be acquired during visits to live animal markets, some in the absence of direct contact with poultry (99-101).

Influenza viral tropism

Influenza virus tropism is determined by a number of different factors, one of which is the hemagglutinin cleavage site of the virus. Human and LPAI viruses are only able to replicate in human tissues in which trypsin-like proteases are available. It has long been known that in humans, these include the respiratory and intestinal tracts (102). In the human respiratory tract, several such proteases, including human airway trypsin-like protease (HAT) and transmembrane protease, serine 2 (TMPRSS2), and prostasin have been identified (103). Both HAT and TMPRSS2 cleave the influenza HA *in vitro*, but evidence of their interaction with influenza virions *in vivo* is lacking, at least in humans

(104). The fact that LPAI and human viruses have been isolated from eye swabs of human conjunctivitis cases suggests that similar that proteases can be found in the eye as well (105). HPAI can and do infect other tissues, and studies in mouse and ferret models have confirmed that the multi-basic cleavage site characteristic of these viruses is required for extrarespiratory or extraintestinal spread (106-108). It is important to note, however, that the presence of a multi-basic cleavage site alone may not be sufficient to enable viral dissemination; not only do many HPAI viruses fail to spread systemically in ferrets, but the addition of a multi-basic cleavage site to a human H3N2 virus failed to confer this ability, though the multi-basic cleavage site containing virus was able to replicate in MDCK cells in the absence of trypsin (109).

A second well-studied determinant of influenza virus tropism in humans is the receptor specificity of the viral hemagglutinin. In 1983, Rogers and Paulson used hemagglutination assays with resialylated erythrocytes to show that as a group, human and swine influenza viruses preferentially bound to α2,6-linked sialic acids whereas avian isolates bound more strongly to α2,3-linked receptors (110). Subsequent studies using more sophisticated techniques and a broader array of viruses and subtypes have supported their findings, establishing as a general rule the preference of human and avian viruses for α2,6 and α2,3 linkages, respectively (111). Lectin-staining of the respiratory tract has been used to show that α2,6 linked sialic acids are much more prevalent that α2,3 ones in the nose, pharynx, and trachea whereas the bronchus contains a mix of the two receptor types, though α2,6 sialic acids predominate in the bronchioles. In the lung, lectin staining indicates that type II pneumocytes primarily, but not exclusively express α2,3 linked sialic acid (112-114). However, a study using mass spectrometry, and therefore able to

observe sialic acids not recognized by lectins, found that α2,3 and α2,6-linked receptors were present in comparable amounts in the lung (115). Studies of viral attachment to histological sections generally show binding patterns consistent with what would be expected based on the lectin data: human viruses bind extensively to the epithelium of the nose, trachea, and bronchi. Less binding is observed in the bronchioles and lung, where attachment to type I pneumocytes is greater than to type II. Both H5N1 and H7N9 viruses attach to type II pneumocytes, alveolar macrophages, and the bronchioles (112, 116-118). H7N9 viruses, which have some affinity for α2,6 receptors also bind to type I pneumocytes, the treachea, and the nasal concha (118-120).

Clinical symptoms and autopsy data indicate that viral replication typically occurs in the regions of the respiratory tract to which the most extensive binding is observed in histological attachment studies: human viruses in upper respiratory tract and avian viruses in the lower respiratory tract. However, this does not necessarily indicate that receptor specificity is the only determinant of tropism within the respiratory tract. Replication studies conducted using *ex vivo* tissues and primary cells differentiated *in vitro* demonstrate that so long as the appropriate receptors are present, they need not predominate in order for productive replication to occur. Human viruses, for example, replicate well in pneumocytes and alveolar macrophages, and H5N1 viruses productively infect nasal and nasopharyngeal cells (114, 121-124). The reasons why such replication is rare *in vivo* (in humans) are unknown, and suggest a need to explore differences between the *in vitro* and *in vivo* environments, both internal and external to the cell. For example, a host restriction factor or factors that interfere with virus production *in vivo* may not be expressed *in vitro*. Alternatively, virions may be prevented from coming into contact with

or infecting susceptible cells *in vivo* by mucins or by the host immune response. The high concentrations of virus used in many *in vitro* studies may also facilitate viral infection and spread in cell types which are minimally susceptible to infection.

In the following chapters, we describe studies of the tropism of both avian and human influenza viruses. First, we investigate the role inoculation method plays in determining cellular tropism *in vitro*. We describe methods for the infection of cultured epithelial cells by exposure to defined quantities of aerosolized influenza virus. Using these procedures, we find that an H7N9 virus is able to replicate productively in primary human alveolar epithelial cells following liquid, but not aerosol inoculation whereas replication of an H5N1 is not affected by inoculation method. In chapter 3, we turn our focus to ocular tropism. While ocular infections with influenza virus are rare and are most strongly associated with avian H7 viruses, study of host-virus interactions in the eye may enhance understanding of tropism in general by illuminating similarities and differences between ocular and respiratory tissues. Because existing studies of influenza infection in ocular cells indicated that the ability to replicate productively *in vitro* did not correspond to signs and symptoms observed in humans, we sought to examine factors which might restrict infection and/or replication in the context of naturally occurring human exposure, but had not previously been tested in the laboratory. We found that culture temperature, inoculation route, and level of cell differentiation only minimally impacted tropism *in vitro*. Human tears, in contrast, not only reduced viral infectivity, but did so in a virus-specific manner, suggesting that conjunctivitis-causing viruses may be unique in their ability to resist inactivation during passage through the tear film rather that in having the capacity to establish productive infection upon reaching the surface of

ocular cells. Collectively, these studies enhance our knowledge of virus tropism in both respiratory and non-respiratory cell types, and identify further avenues which warrant continued investigation.

Chapter 2: *In vitro* exposure system for study of aerosolized influenza virus

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This work was published in *Virology*

Article citation:

Creager HM, Zeng H, Pulit-Penaloza JA, Maines TR, Tumpey TM and Belser JA. 2017. *In vitro* exposure system for study of aerosolized influenza virus. Virology 500: 62-70.

Abstract

Infection of adherent cell monolayers using a liquid inoculum represents an established method to reliably and quantitatively study virus infection, but poorly recapitulates the exposure and infection of cells in the respiratory tract that occurs during infection with aerosolized pathogens. To better simulate natural infection *in vitro*, we adapted a system that generates viral aerosols similar to those exhaled by infected humans to the inoculation of epithelial cell monolayers. Procedures for cellular infection and calculation of exposure dose were developed and tested using viruses characterized by distinct transmission and pathogenicity phenotypes: an HPAI H5N1, an LPAI H7N9, and a seasonal H3N2 virus. While all three aerosolized viruses were highly infectious in a bronchial epithelial cell line (Calu-3) cultured submerged in media, differences between the viruses were observed in primary human alveolar epithelial cells and in Calu-3 cells cultured at air-liquid interface. This system provides a novel enhancement to traditional *in vitro* experiments, particularly those focused on the early stages of infection.

Introduction

Infection of adherent cell monolayers using a liquid inoculum represents an established method to reliably and quantitatively study virus infection. Relatively straightforward and inexpensive, this method allows for the frequent collection of viral samples and the testing of a variety of experimental conditions and discrete cell types including those of human origin. Unfortunately, traditional *in vitro* replication studies poorly recapitulate the exposure and infection of cells in the respiratory tract that occurs during natural exposure to aerosolized pathogens. Not only does infection occur while cells' apical surface is immersed in liquid, but at typical cell densities, the often-used "low" multiplicity of infection (MOI) of 0.01 corresponds to a dose of over a thousand PFU per square centimeter.

Available evidence suggests that in the case of aerosol transmission, natural human influenza infection is likely initiated by substantially fewer particles. Studies of infected patients found low viral concentrations in aerosols generated by breathing, coughing, and/or sneezing (Fabian et al., 2008; Milton et al., 2013; Yang et al., 2011), and fewer than five $TCID_{50}$ are capable of initiating symptomatic infection in experimentally exposed volunteers (Alford et al., 1966). Similar results have been observed in the ferret model; these animals can be infected with fewer than ten PFU and subsequently exhale under five PFU per minute (Gustin et al., 2015; Gustin et al., 2011; Gustin et al., 2013; Roberts et al., 2011). Using a library of barcoded viruses, Varble et al. found that respiratory droplet transmission between ferrets involved only single-digit numbers of virions (Varble et al., 2014). Reports of A(H7N9) cases developing subsequent to patient visits to live bird markets despite lack of poultry contact, and the

detection of virus in air sampled from such markets, indicate that zoonotic infection may also occur after human exposure to low quantities of aerosolized virus (Li et al., 2015; Liu et al., 2014; Zhou et al., 2016).

In order to better study the effects of potentially damaging aerosols on human cells, the toxicology field has begun to expose cultured respiratory epithelial cells to aerosolized, rather than liquid-suspended, chemical and particulate matter. Cells have been shown to be more sensitive to the effects of the former (Bitterle et al., 2006; Raemy et al., 2012). In these studies, aerosol concentration can be measured by the use of optical or gravimetric methods. These types of methods are not effective for the measure of virus-containing aerosols, however, because they detect liquid droplet nuclei rather than the virus within them, and cannot differentiate between infectious and non-infectious virions. Microbiologists have developed aerosolization systems to overcome these challenges, and have used them for experimental infections of animals and to study the effect of environmental conditions on viability of numerous pathogens including *Mycobacterium tuberculosis*, *Bacillus anthracis*, measles virus, and influenza virus (Clark et al., 2011; Gustin et al., 2011; Lemon et al., 2011; Savransky et al., 2013). This work has provided important insights into the intra- and inter-host spread of these pathogens by facilitating the observation and manipulation of near-natural infection within a controlled laboratory environment. However, despite the frequent employment of *in vitro* studies to complement animal experimentation, use of an aerosol system for *in vitro* infection with any pathogen has not, to our knowledge, been previously described.

We combined aspects of the toxicological and microbiological approaches to establish a novel method to expose adherent mammalian cell monolayers in air-liquid

interface to defined quantities of aerosolized influenza virus and compared this with traditional liquid inoculation. In order to most effectively mimic the conditions of natural infection, we explored the use of very low MOI infection and culture techniques designed to promote cell differentiation in conjunction with virus aerosolization. Using highly pathogenic avian influenza (HPAI), low pathogenic (LPAI), and seasonal influenza viruses, we demonstrate that infection of respiratory epithelial cells with physiologically low concentrations of aerosolized virus can be successfully recreated inside the laboratory. In conjunction with research using animal models, these techniques facilitate a closer study of the infectivity of aerosolized influenza virus in the context of human infection. The approach described here is not restricted to influenza virus and would also be applicable to the study of other respiratory viruses of public health concern.

Materials and Methods

Viruses

Influenza A viruses were propagated in the allantoic cavity of 10-day-old embryonated hens' eggs and titered via standard plaque assay using Madin-Darby canine kidney (MDCK) cells as previously described (Maines et al., 2005; Zeng et al., 2007). All experiments were conducted under biosafety level 3 containment, including enhancements as required by the U.S. Department of Agriculture and the Federal Select Agent Program (Chosewood et al., 2009).

Cell culture and liquid inoculations

The bronchial epithelial cell line Calu-3 (ATCC) was cultured as previously described (Zeng et al., 2007). Primary human alveolar epithelial cells (Cell Biologics) were cryopreserved at passage 3, then grown and expanded per manufacturer's instructions. All cells were seeded on 24 mm diameter (6-well format) or 12 mm diameter (12-well format) semipermeable membrane inserts with a 0.4μm pore size (Corning) and grown to confluence under submerged conditions. After reaching a transepithelial resistance of >1,000 Ω^2 (Zeng et al., 2007), apical media was removed from selected Calu-3 cells to create an air-liquid interface (ALI), which was maintained for three weeks to facilitate cell differentiation and the establishment of a mucin layer.

Prior to inoculation, apical media (if present) was removed from the cell monolayer and cells cultured under submerged conditions were washed to remove serum present in culture media. Liquid inoculation was performed using 300μL of virus, diluted as specified in the results, and incubated on the cell surface for one hour before washing. After infection, cells were cultured in cell type-specific serum-free medium to which 1μg/mL N-p-tosyl-ʟ-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich) was added for alveolar cell cultures. Aliquots of apical culture supernatant or wash media (incubated atop cells cultured at ALI for 20 minutes) were collected at the indicated times post-infection (p.i.) and immediately frozen at -80°C until titration. Growth curves were generated and analyzed using Prism 6.0.7 (GraphPad Software Inc.).

Aerosol inoculations

The automated bioaerosol system used for all experiments has been previously described in detail (Gustin et al., 2011; Hartings and Roy, 2004) and all conditions were

maintained here unless specified otherwise. Briefly, virus suspended in a solution of PBS-0.03% (w/v) BSA was aerosolized using a three-jet Collision nebulizer (BGI, Inc.) and passed through an exposure chamber at a rate of 20 L/min. Cells placed inside the exposure chamber on a wire mesh shelf were inoculated under air-liquid interface conditions (Fig. 1). Using the AeroMP (Biaera Technologies) aerosol management platform, aerosol exposures were conducted at 21°C and 50% relative humidity for 15 min followed by a 5 min purge to allow evacuation of the aerosolized virus from the chamber (Gustin et al., 2011). Prior to *in vitro* exposure, spray factor (SF) values were determined for stocks of all viruses to estimate the starting virus concentration in the nebulizer needed to obtain a desired quantity of virus in the aerosol. A Biosampler (SKC Inc) was used to quantify the virus actually aerosolized within the chamber during each exposure. Following aerosol exposure, membrane inserts were transferred to clean 6-well or 12-well plates and serum-free medium was added to apical and basolateral compartments as described above; cells maintained under ALI conditions had medium added to the basolateral compartment only. Confirmation of cell viability during aerosol exposure was performed using the WST-1 cell proliferation reagent (Roche Applied Science), according to manufacturer's instructions, with four independent samples tested for each condition.

Quantitation of Exposure and Infectious Doses

The total number of infectious virions passed through the chamber during the exposure session (N_{cham}) was calculated as

$$N_{cham} = \frac{(C_{samp})(V_{samp})(Q_{cham})}{Q_{samp}}$$

where C_{samp} is the concentration of virus in the sampler, V_{samp} is the volume of media in the sampler, and Q_{cham} and Q_{samp} represent the flow rates of chamber and sampler, respectively (see supplemental methods for derivation). N_{cham} was multiplied by the ratio of the surface area of each transwell (*SA*) to the cross-sectional area of the chamber (*XA*) to yield exposure dose (*ED*). ID_{50}s were calculated according to the method of Reed and Muench, with the proportional distance multiplied by $log_{10}(dose\ above\ 50\%)/log_{10}(dose\ below\ 50\%)$ to account for the deviation of exposure doses from exact 10-fold dilutions (Reed and Muench, 1938). Variability around the mean exposure dose was quantified using a binomial model with *n* viable virions passing through the chamber and a probability of success (virion lands on well) equal to *SA/XA*. Upper and lower critical values at the 95% confidence level were calculated using R 3.2.3 (R Foundation for Statistical Computing); the true exposure dose for any particular well has a 95% chance of falling between these two values. The cumulative probability distribution indicated that the minimum dose had to be three or greater for 95% of all wells to be inoculated with at least one virion. In order minimize the chance of including a well not exposed to any virus, we therefore aimed not to use mean exposure doses under 5 PFU. When this did occur due to variations in aerosolization efficiency, the minimum 50% infectious dose is reported as ≤ 3 PFU.

Real-time RT-PCR.

Total RNA was extracted from mock-infected or virus-infected cell monolayers after removal of supernatant using the RNeasy mini kit (Qiagen). RT-PCR was performed with a QuantiTect SYBR green RT-PCR kit (Qiagen) in duplicate reactions

from duplicate samples using an influenza A virus M1 gene primer set (Zeng et al., 2007). Influenza virus M gene RNA copy numbers were extrapolated using a standard curve based on samples of known M gene copy number.

Results

System establishment and exposure dose determination

Rather than use the type of exposure system described in studies of cellular responses to aerosolized particulate matter (eg. CULTEX Radial Flow System), we employed one intended for use in animal infection. Previously optimized for use with influenza virus (Gustin et al., 2011), this system and its components are designed to maintain the viability of biological aerosols. This system features a large exposure chamber, which allows for simultaneous exposure of several plates of cells, facilitating comparisons of different cell types or growth conditions between uniformly exposed wells (Fig. 1).

In vitro inoculations of cells grown on semi-permeable membrane inserts (transwells) were conducted as follows: apical media (if present) was removed immediately prior to exposure, then plates holding the inserts and basolateral media were placed in the chamber and exposed to ten-fold serial dilutions of aerosolized virus for a duration of 15 minutes. Following exposure, inserts were transferred to a sterile tissue culture plate containing fresh basolateral media and apical media was replaced. Mock infection led to no significant decrease in cell viability (data not shown), indicating that the presence of basolateral media was sufficient to keep the cells from drying out during exposure.

The ability to quantitate the dose to which an animal or cell monolayer is exposed is a critical component of experiments utilizing aerosol-based exposure systems (Hartings and Roy, 2004), and is necessary for comparison of aerosol infections with those conducted by the more traditional liquid route. We found that titration of virus collected in wells of media placed in the chamber alongside the cells was insufficiently sensitive to reliably quantify the low doses to which each monolayer was exposed (data not shown), possibly because deposition efficiency differed between the cell surface and the liquid media. We therefore modeled our approach on that used in aerosol inoculation of animals. Presented dose for an animal can be expressed as the total number of infectious virions passing through the chamber during an exposure session multiplied by the ratio of the volume of aerosol inhaled by the animal to the total volume of aerosol passed through the chamber (see supplemental methods for derivation). For *in vitro* quantification, we substituted the ratio of the surface area of each transwell insert to the cross-sectional area of the chamber for the ratio of inhaled to total aerosol volume. Because the efficiency of particle deposition on the cell surface was estimated to be approximately 100%, presented dose and exposure dose were considered equivalent. No correlation between plate position within the chamber or well position within the plate and virological outcome was observed.

Validation of experimental approach

Initial characterization studies were conducted using the Calu-3 human bronchial epithelial cell line. This cell type is relevant to respiratory infection and has previously been shown to support replication of a variety of influenza A viruses, though published

studies have typically used MOIs of 0.01 (equivalent to 10,000 $TCID_{50}$ or PFU per 24mm well) or higher (Zeng et al., 2007; Zhou et al., 2011). In order to generate comparison data for aerosol infections, we first conducted an analogous experiment using traditional liquid inoculation at a wide range of doses. Two influenza viruses known to replicate with high efficiency (A/Thailand/16/2004 [Thai/16, HPAI A(H5N1)] and A/Anhui/1/2013 [Anhui/1, LPAI A(H7N9)], both isolated from fatal human cases) were serially diluted and used to inoculate quintuplicate wells. Because of the potential for both random and systemic error in making repeated serial dilutions, we titrated all inocula, enabling us to more precisely estimate the number of infectious virions to which each well was exposed. RNA was collected from the cell monolayers of two wells 24 hours post-inoculation and assayed for the presence of viral nucleic acid via RT-PCR with primers specific to the M1 gene. Growth kinetics in the remaining three wells were monitored by titration of cell supernatants collected between 2 and 96 hours post-infection (Fig. 2). At inoculum doses above our limit of detection (10 PFU/mL or 3 PFU/well), both viruses consistently infected all replicate wells and replicated to high titer, though growth was somewhat delayed at lower inoculation doses relative to higher ones (Figs. 2, 3B). We also observed robust replication in cultures inoculated with approximately 1 PFU (dose estimated from serial dilution) of Anhui/1 virus. At doses of less than one PFU, infection was infrequent, characterized by low titers and undetectable levels of viral nucleic acid in the cell monolayer 24 hours after inoculation.

Aerosol experiments were conducted with three viruses chosen to represent a diversity of mammalian *in vivo* pathogenicity and transmissibility phenotypes: Thai/16, Anhui/1, and A/Panama/2007/99 (Panama/99, seasonal A(H3N2)). All three viruses

infected Calu-3 cells with high efficiency following aerosol exposure (Fig. 3). We observed rates of productive infection comparable to those seen after inoculation using a liquid suspension, with 50% infectious doses (ID_{50}s) for all three viruses of under five PFU. High peak viral titers (10^8 PFU/ml) were detected in the supernatant regardless of exposure dose, though, as with liquid inoculum, replication was delayed at lower inoculation doses (Fig. 3). Duplicate cultures were incubated post-exposure at 33°C, a temperature thought to represent that of the mammalian upper respiratory tract, after infection to see whether infectivity was temperature-dependent. We found that while 24-hour titers of the two avian viruses were slightly lower at this temperature than at 37°C, infectivity of these cultures was reduced only for Panama/99 virus, and only slightly (ID_{50} of 12 vs $\leq$ 3 PFU).

The concordance in infectious dose between aerosol and liquid inoculations suggested that our calculated exposure dose for each well accurately represented the average number of virions to which a well was truly exposed. To confirm this, we conducted a series of exposures at doses near 1 PFU (range 0.02 to 7 PFU) per well. In light of our liquid exposure data with Thai/16 and Anhui/1, we reasoned that if the calculated exposure doses were accurate, the majority of Calu-3 wells should be infected with either of these viruses at doses $\geq$1 PFU, whereas few wells would be infected at doses <1 PFU. Consistent infection at calculated doses under 1 PFU would indicate that the true exposure dose was higher than the calculated one. Conversely, infrequent infection at doses $\geq$1 PFU would suggest that the actual exposure dose was less than the calculated one. In order to conduct a quantitative analysis, we determined the number of wells we would expect to become infected after each set of exposures using a Poisson

binomial model (Hong, 2013). We statistically compared the expected number of infected wells (based on our calculated exposure dose) to the number of wells observed to be infected in our experiment (reflective of actual exposure dose) (Table 1). Even though our tests were highly powered (≥ 99% chance of detecting a half-log difference between true and calculated exposure doses if a difference was present), we did not detect such a difference for either of the viruses tested ($p>0.05$). This suggested that our estimation of 100% deposition efficiency did not compromise our exposure dose estimates.

Infection of primary human alveolar cells

Having validated our methodology using a transformed cell line, we next investigated the ability of low-dose aerosols to infect primary human alveolar epithelial cells. Seasonal influenza A viruses are typically restricted to the upper respiratory tract during infection of humans and mammalian models, while avian viruses are often detected in the lungs. However, multiple *in vitro* studies have found primary human alveolar cells to be permissive for the replication of human influenza viruses (Weinheimer et al., 2012; Yu et al., 2011). We hypothesized that this discrepancy might be an artifact caused by the artificial nature of the liquid inoculum used in the *in vitro* studies. After confirming that liquid-based inoculation with Thai/16, Anhui/1, and Panama/99 viruses at an MOI of 0.01 led to productive replication in primary human alveolar cells (Figs. 4A and 4C), we exposed the alveolar cells to multiple concentrations of the same three viruses, this time by the aerosol route. Thai/16 virus was highly infectious, replicating productively in wells exposed to an average of ≤ 3 PFU (MOI = $2.6x10^{-6}$). In contrast, replication of Panama/99 or Anhui/1 viruses could not be detected at any of the doses tested following aerosol inoculation (Fig. 4A). Repetition of the

highest-dose aerosol inoculation confirmed the absence of detectable replication of Anhui/1 virus (Fig. 4C). Low levels of Panama/99 virus were present in the cell supernatant of all three triplicate wells, but in all cases, titers peaked 24 hours post-infection and declined thereafter, indicating unsustained or unproductive replication. To determine whether the lack of replication was a product of inoculation route or dose, we exposed cells to a liquid inoculum at an MOI (~0.001) equivalent to that of our most concentrated aerosol inoculation. Growth kinetics were similar to those observed after the 0.01 MOI infections, suggesting that the lack of replication in a permissive cell type could be attributed to the aerosol delivery of the virus and not to its low concentration.

Infection of cells cultured under air-liquid interface

In order to reach susceptible cells, inhaled pathogens must penetrate the mucus layer coating the airway epithelium. The mucus layer and epithelium can be simulated *in vitro* by culturing respiratory cell lines or primary cells at air-liquid interface (ALI: media is present only on the basolateral side of the monolayer, with the apical side left exposed to air). Culture at ALI causes cells to differentiate and form a pseudostratified, columnar epithelium, comprising multiple cell types, including mucus-secreting goblet cells (Kreft et al., 2015). However, it has not previously been possible to recreate the interaction of aerosolized pathogens with respiratory mucus in an *in vitro* setting. We therefore compared the susceptibility of Calu-3 cells grown at ALI and liquid-liquid interface (LLI: media present on apical and basolateral sides) to infection with aerosolized virus. Infectivity of Calu-3 cells cultured at ALI for three weeks (sufficient to induce substantial mucus production (Haghi et al., 2010; Kreft et al., 2015)) varied by virus (Fig. 5):

Panama/99 infectivity was only slightly abrogated (ID_{50} increased from ≤ 3 to 7) whereas Thai/16 and Anhui/1 were over 25 times less infectious in the ALI-cultured cells than those cultured at LLI. When cells were successfully infected with any of the viruses, replication was delayed and reached lower titers in ALI cultures as compared to LLI (Fig. 5). Together, these data demonstrate that the capacity for aerosolized inocula to infect cultures of differentiated human airway cells is abrogated but not eliminated by the presence of a mucin layer.

Discussion

While a growing body of work describes the characteristics of aerosolized microbes, the interaction of pathogen-laden aerosols with the airway epithelium has not been specifically examined. We demonstrate here that infection of adherent cell monolayers by aerosol is feasible, and we describe and validate a straightforward method for the calculation of exposure dose, which facilitates comparison between infections using liquid and aerosol inocula. While *in vitro* replication studies typically measure viral titers over time after infection with a single MOI, we found that measuring viral infectivity at a variety of MOIs was more informative.

Measurements of infectivity may also be more relevant to virus transmission. Interestingly, we found that culture of Calu-3 cells at 33°C post-aerosol-inoculation did not significantly reduce the ability of any of the viruses tested to establish infection. While we did not perform a liquid comparison, we would expect comparable results given the consistency of replication of the tested viruses in this cell type. It is important to note, however, that all three viruses have a lysine at position 627 of the PB2 gene

rather than a glutamate, which is known to restrict replication at this temperature (Neumann and Kawaoka, 2015). More significant effects on infectivity might be observed with viruses that have a glutamate at this position or in other cell types.

Studies utilizing very low MOI may play an important role in advancing our understanding of influenza virus transmission by simulating *in vitro* the conditions under which infection is first established in a new human host. At decreased MOI, for example, the rate at which semi-infectious particles are able to replicate synergistically through cellular co-infection is reduced (Fonville et al., 2015), but the diminished proliferation of defective interfering particles may lessen innate immune activation, thereby allowing for increased viral replication. Indeed, previously published work with influenza and other viruses describes differences in viral replication at high and low MOI (Aggarwal et al., 2011; Huh et al., 2008; Miller et al., 1994). We have demonstrated here the feasibility of studies using very low MOI; though we observed only subtle changes in replication kinetics as MOI decreased, similar across all viruses tested, more pronounced effects may be observed with other viruses, cell types, and/or culture conditions (*e.g.* temperature).

Droplet nuclei (<5μm) containing virus, unlike larger (>5μm) droplets or virus present on surfaces, reaches the lower respiratory tract when inhaled (BeruBe et al., 2009). We were therefore particularly interested in the ability of aerosolized virus to infect primary human alveolar cells. We found that both the seasonal virus Panama/99 and an outbreak-associated A(H7N9) virus, Anhui/1, replicated productively in primary human pneumocytes after inoculation via the liquid, but not aerosol, route whereas the HPAI virus Thai/16 was highly infectious and replicated to high titer regardless of inoculation method. For Panama/99 virus, these *in vitro* results are consistent with both

ferret studies, which do not detect virus replication in the lungs, and with human seasonal virus infections, which are typically limited to the upper respiratory tract. In contrast, severe human A(H7N9) cases have been characterized by symptoms of lung infection (Chen et al., 2013; Gao et al., 2013; Hu et al., 2013; Yang et al., 2014; Yu et al., 2013b), and virus has been detected in the lungs of experimentally infected animals (Belser et al., 2013; de Wit et al., 2014; Gabbard et al., 2014; Watanabe et al., 2013; Xu et al., 2014; Zhang et al., 2013; Zhu et al., 2013). Our findings raise the possibility that the development of viral pneumonia associated with H7N9 virus develops not upon initial exposure, but subsequent to viral spread from adjacent tissues, and warrant further investigation regarding the dynamics of H7N9 virus infection throughout the respiratory tract. The need for such spread may provide a window of opportunity for the immune system to restrict the virus before it causes severe disease, which would explain the apparent prevalence of clinically inapparent and mild infection with this virus (Chen et al., 2014; Ip et al., 2013; Yu et al., 2013a).

Using the Calu-3 cell line, we demonstrated that growth under ALI conditions reduced the efficiency of both initial infection and subsequent viral replication. The high viscosity of mucus and abundance of virus-binding sialic acids may limit the diffusion of virus between cells, thereby reducing viral titers. Notably, abrogation in infectivity resulting from culture at ALI was more pronounced with the two avian viruses tested than the seasonal virus Panama/99. This finding is consistent with the hypothesis that respiratory mucus serves as an important barrier to the ability of avian influenza viruses to transmit between humans, possibly because their specificity for α2,3-linked sialic acids makes them more susceptible to binding and entrapment by mucus, which some studies

have suggested contains glycans primarily in the α2,3 conformation (Baum and Paulson, 1990; Couceiro et al., 1993). Use of reverse genetics techniques to compare viruses differing only in the sialic binding preferences of the hemagglutinin and/or neuraminidase proteins will allow for further investigation of this phenomenon.

Aerosol inoculation, particularly when used in conjunction with increasingly sophisticated techniques for *in vitro* cell culture, offers a unique opportunity to study virus-cell interactions in an environment resembling that of the human respiratory tract. Our studies suggest that aerosol inoculation may enhance studies of viral tropism and improve our understanding of the effects of environmental conditions on the ability of influenza virus to initiate infection. Continued investigation regarding the role of inoculation route in viral binding and entry processes will further our understanding of the infectivity of influenza viruses with distinct phenotypes. In addition to influenza, the methods outlined here could be used for the study of other respiratory viruses such as severe acute respiratory syndrome (SARS) virus, varicella zoster virus (VZV), and measles virus, as well as the risk assessment of novel pathogens. The ability to combine aerosol inoculation with the benefits of *in vitro* study, notably the ability to study specific cell types in isolation, will facilitate a greater understanding of the infectivity and tropism of respiratory pathogens of public health concern.

Acknowledgements

We thank the China CDC for facilitating access to virus, Justin Hartings for technical assistance, and Alissa Eckert for graphical assistance. H.M.C. and J.A.P. were supported by the Oak Ridge Institute for Science and Education. The findings and

conclusions in this report are those of the authors and do not necessarily reflect the views of the funding agency.

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Tables

Table 1. Concordance between observed and expected infection rates of wells exposed to doses near 1 PFU

Exposure dose[a]	Virus	Observed # infected[b]	Expected # infected[c]	*p*-value[d]	Power[e]
>1 PFU	Thai/16	9/12	≥ 10.5/12	0.17	0.99
	Anhui/1	15/18	≥ 16.5/18	0.18	1
< 1 PFU	Thai/16	8/24	≤ 8.0/24	0.58	1
	Anhui/1	0/18	≤ 6.1/18	1	1

[a]Calculated exposure dose, as described in the text.

[b]Number of experimentally infected wells, as determined by ≥ 10 PFU/mL in the supernatant at both 72 and 96 hours post-inoculation.

[c]Number of wells expected to be infected based on calculated exposure dose. Determined using a Poisson binomial distribution where n = the number of exposed wells and p = the probability of an exposure dose of ≥ 1 PFU, where exposure dose follows a binomial distribution where n = total virions and p = well surface area/chamber cross-sectional area.

[d]One-sided *p*-value reflecting the statistical significance of the difference between the number of wells expected and observed to be infected. Calculated using the Poisson binomial distribution described above.

[e]Power to detect a half-log or greater difference between calculated and actual exposure dose.

Figures

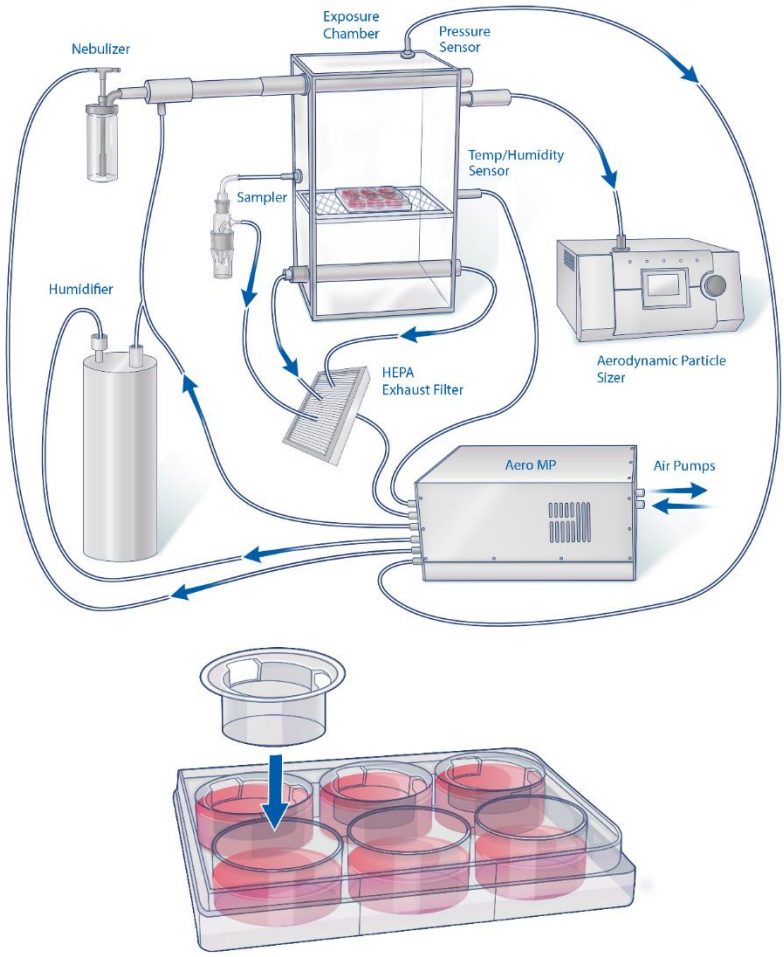
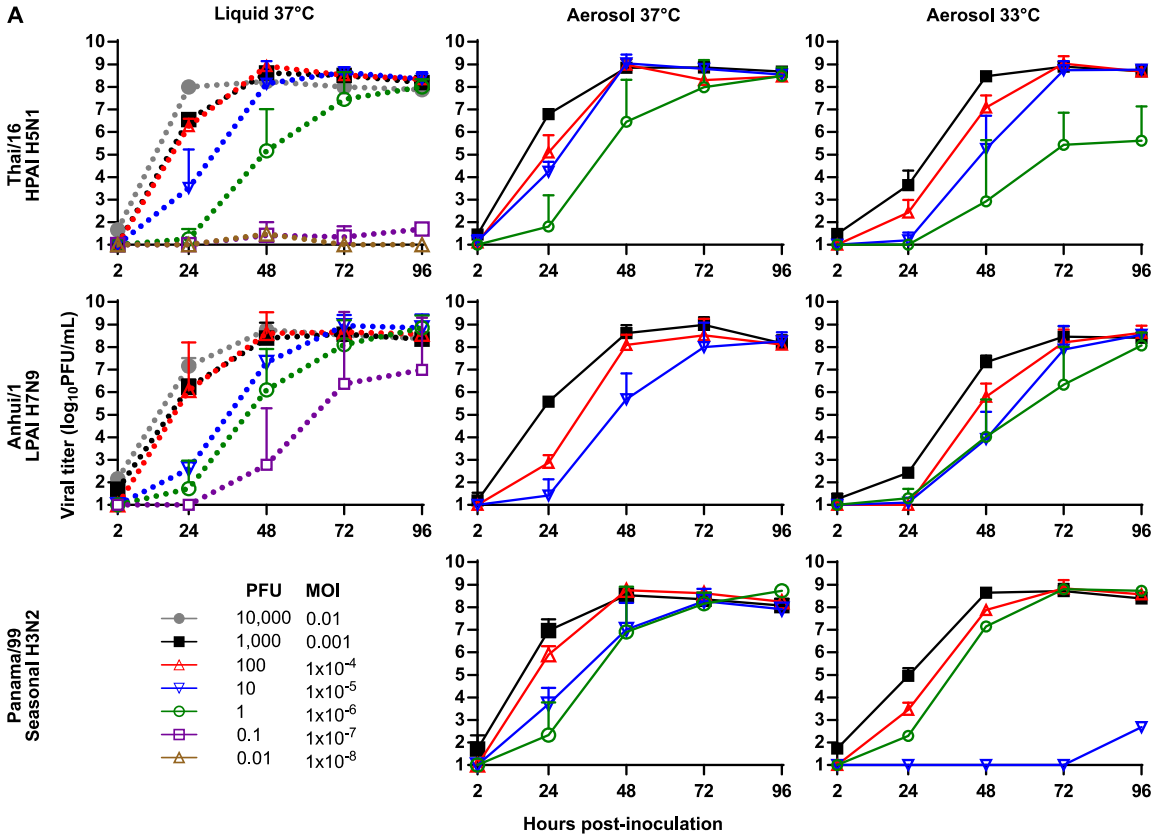


Figure 1. Graphic representation of aerosol system for *in vitro* use

Depiction of human cells cultured on transwell inserts and exposed to aerosolized influenza virus using a previously characterized system (Gustin et al., 2011). Cell culture dishes rest in the exposure chamber on a wire shelf under air-liquid interface conditions for the duration of the exposure. Inset, individual transwell inserts are transferred to sterile plates once removed from the exposure chamber.



B

Legend		Liquid				Aerosol					
All viruses		Thai/16		Anhui/1		Thai/16		Anhui/1		Panama/99	
PFU	MOI	PFU	MOI	PFU	MOI	PFU	MOI	PFU	MOI	PFU	MOI
10,000	0.01	9,150	0.009	13,650	0.01	–	--	--	--	–	--
1,000	0.001	750	0.0008	1,320	0.001	2,410	0.002	2,055	0.002	4,110	0.004
100	$1x10^{-4}$	93	$9x10^{-5}$	156	$2x10^{-4}$	355	$4x10^{-4}$	262	$3x10^{-4}$	1,065	0.001
10	$1x10^{-5}$	9	$9x10^{-6}$	27	$3x10^{-5}$	9	$9x10^{-6}$	15	$2X10^{-5}$	12	$1x10^{-5}$
1	$1x10^{-6}$	3	$3x10^{-6}$	12	$1x10^{-5}$	2	$2x10^{-6}$	1	$1X10^{-6}$	4	$4x10^{-6}$
0.1	$1x10^{-7}$	< 3	$< 3x10^{-6}$	< 3	$< 3x10^{-6}$						
0.001	$1x10^{-8}$	< 3	$< 3x10^{-6}$	< 3	$< 3x10^{-6}$						

Figure 2. Replication of influenza A viruses in Calu-3 cells

A) Calu-3 cells were infected by the traditional liquid route (dashed line) or the aerosol route (solid line) at the target MOI with the viruses shown, and cultured at 37°C or 33°C. Culture supernatants were collected at the indicated times p.i., and titers were determined by standard plaque assay to quantify infectious virus. The limit of detection was 10 PFU.

Error bars indicate standard deviation. Lines represent positive wells (infectious virus detected at two sequential timepoints or at 96 hours alone, 3/3 unless otherwise noted) only. Cultures with 2/3 positive wells: Thai/16 33°C aerosol $1x10^{-6}$, liquid $1x10^{-7}$; Anhui/1 33°C aerosol $1x10^{-6}$, liquid $1x10^{-7}$; Panama/99 37°C aerosol $1x10^{-6}$. Cultures with 1/3 positive wells: Thai/16 37° liquid $1x10^{-8}$; Panama/99 33°C aerosol $1x10^{-5}$ and $1x10^{-6}$. B) Exact inoculum dose (PFU) and MOI for each infection shown in panel.

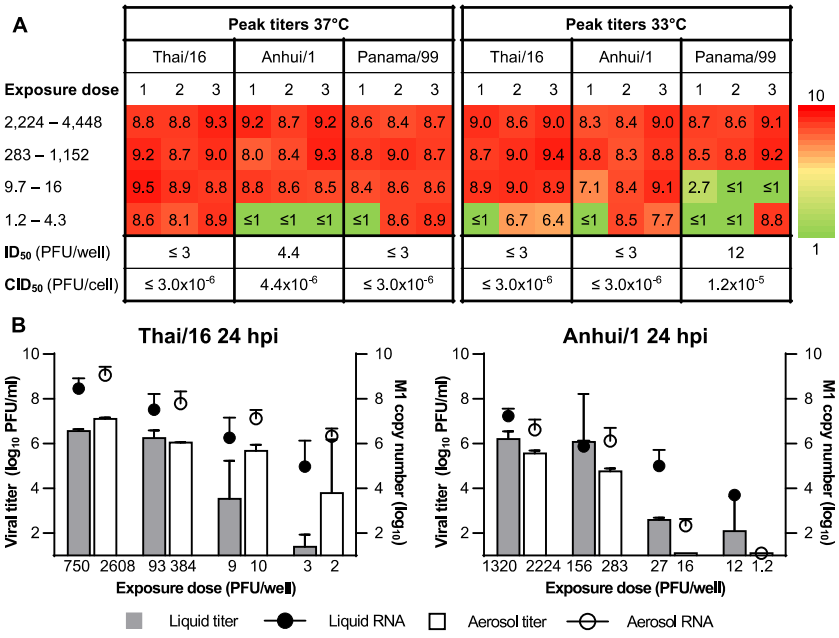


Figure 3. Infection and replication in Calu-3 cells after aerosol exposure

A) Peak viral titers detected in each well inoculated via the aerosol route. Titers (log_{10}PFU/mL) are provided for each well that showed evidence of productive replication, defined as infectious virus detected at two sequential timepoints or at 96 hours alone (full replication curves are shown in Fig. 2). Exposure dose (PFU) varied slightly between viruses and is therefore listed as a range. Exact exposure doses for each virus are listed in Table S1. Cells cultured at 33 and 37°C were exposed concurrently. CID_{50} indicates the 50% cellular infectious dose, or MOI required to achieve 50% infectivity, calculated by dividing ID_{50} by the cell number. Limit of detection was 10 PFU. B) Comparison of viral supernatant titer (left Y axis) and M copy number (right Y axis) present in the cell monolayer between aerosol and liquid inoculation at 24 hours p.i. Cells were cultured at 37°C. Supernatants were collected immediately prior to lysis of the cell monolayer for RNA collection. Each parameter is expressed as mean ± standard

deviation of two independent wells. Limit of detection for M segment RNA was 10 copies.

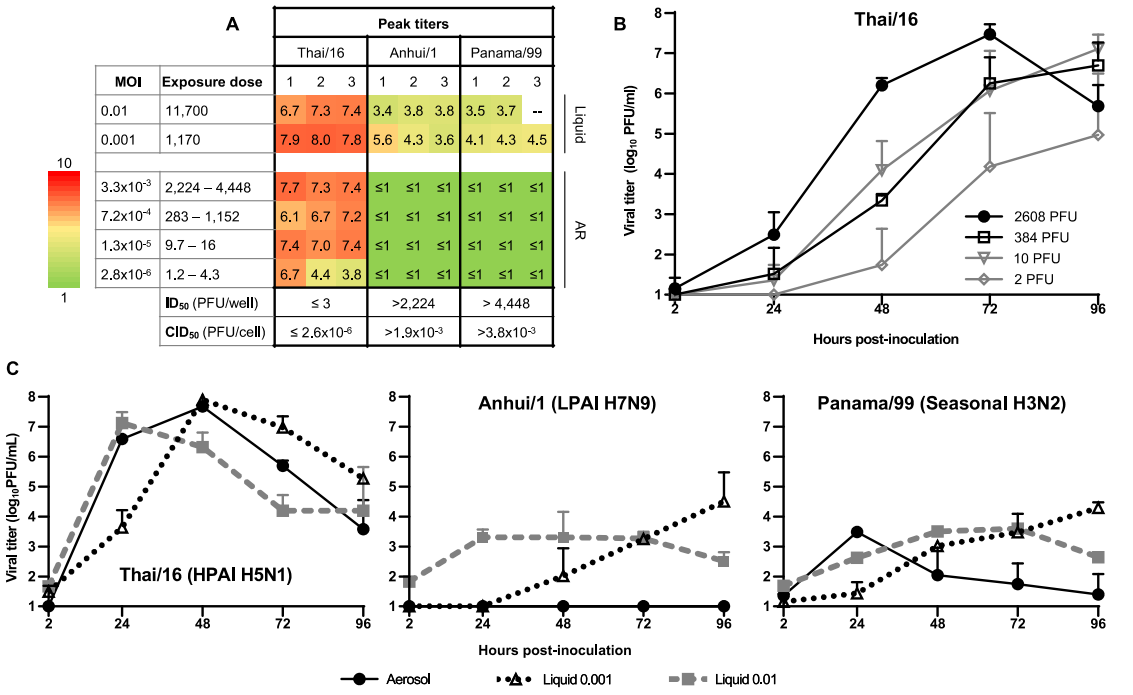


Figure 4. Replication of influenza A viruses in primary human alveolar epithelial cells

A) Peak viral titers detected in each well inoculated via the aerosol route, as described in the legend for Fig. 1A. B) Replication curves for Thai/16 aerosol inoculations shown in panel A. Titers shown represent mean ± standard deviation of three wells. C) Comparison of replication kinetics subsequent to aerosol or liquid inoculation of human primary alveolar epithelial cells. Cells were infected by the aerosol route (solid black line), or by the traditional liquid route (dotted line) at an MOI of 0.01 (gray) or 0.001 (black). Aerosol MOIs were 0.002, 0.0006, and 0.002 for Thai/16, Anhui/1, and Panama/99 viruses, respectively. Culture supernatants were collected at the indicated times p.i., and titers were determined by standard plaque assay to quantify infectious virus. The limit of detection was 10 PFU. Mean ± SD from triplicate cultures (duplicate for Panama/99 liquid inoculum, 0.01 MOI) is shown.

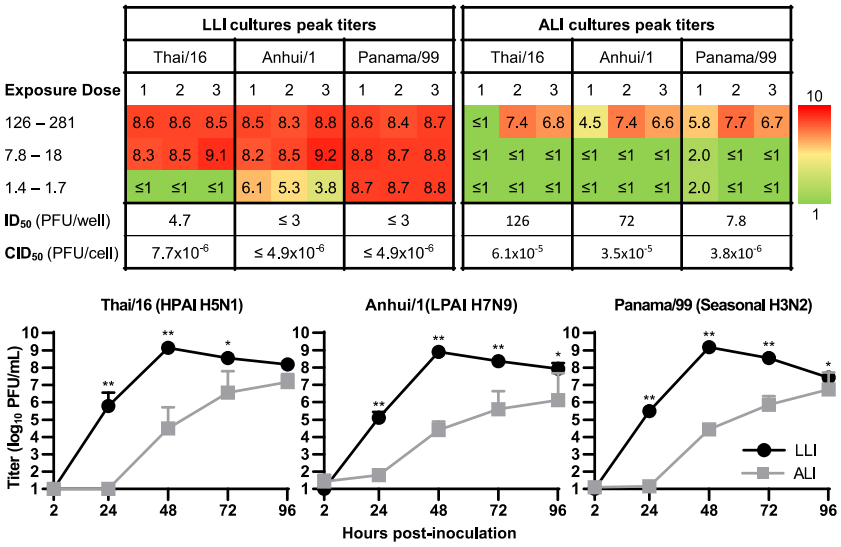


Figure 5. Comparison of viral infectivity and replication between cells cultured at liquid-liquid (LLI) or air-liquid (ALI) interface

A) Peak viral titers detected in each well inoculated via the aerosol route, as described in the legend for Fig 3A. Exact exposure doses are listed in Table S2. Cells cultured at LLI and ALI were exposed concurrently and therefore have identical exposure doses. B) Replication curves from wells exposed to 116-260 PFU shown in panel A. Culture supernatants were collected at the indicated times p.i., and titers were determined by standard plaque assay to quantify infectious virus. The limit of detection was 10 PFU. Titers shown represent mean ± standard deviation of three wells (two in the case of Thai/16). Two-way ANOVA showed that overall titer differences between cells cultured at LLI and ALI were statistically significant ($p<0.05$). For each virus, titers from LLI and ALI wells were compared at each individual timepoint (p-values were adjusted for multiple comparisons using the Bonferroni correction): * indicates $p < 0.05$, ** indicates $p < 0.001$

Supplementary Materials

Supplementary Table 1. Individual values comprising the exposure dose range listed in Figures 3 and 4

Exposure dose range[a]	Virus	Exposure dose[a]	Critical values[b]		MOI[c]	
			Lower	Upper	Calu-3	Alveolar
2,224 - 4,448	Thai/16	2,608	2,509	2,708	$4x10^{-3}$	$2x10^{-3}$
	Anhui/1	2,224	2,132	2,316	$3x10^{-3}$	$2x10^{-3}$
	Panama/99	4,448	4,318	4,578	$7x10^{-3}$	$4x10^{-3}$
283 – 1,152	Thai/16	384	346	423	$6x10^{-4}$	$3x10^{-4}$
	Anhui/1	283	251	316	$4x10^{-4}$	$2x10^{-4}$
	Panama/99	1,152	1,086	1,219	$2x10^{-3}$	$1x10^{-3}$
9.7 - 16	Thai/16	9.7	4	16	$1x10^{-5}$	$8x10^{-6}$
	Anhui/1	16	9	24	$2x10^{-5}$	$1x10^{-5}$
	Panama/99	13	6	20	$2x10^{-5}$	$1x10^{-5}$
1.2 - 4.3	Thai/16	2.0	0	5	$3x10^{-6}$	$2x10^{-6}$
	Anhui/1	1.2	0	4	$2x10^{-6}$	$1x10^{-6}$
	Panama/99	4.3	1	9	$7x10^{-6}$	$4x10^{-6}$

[a]Expressed as PFU/well

[b]Values for a 95% confidence level assuming that presented dose follows a binomial distribution with $N = N_{cham}$ and $p = SA/XA$. The true presented dose for any given well has a 95% chance of falling between these two values.

[c]Multiplicity of infection (MOI) calculated for alveolar or Calu-3 cells exposed to virus, expressed as PFU/cell

Supplementary Table 2. Individual values comprising the exposure dose range listed in Figure 5

Exposure dose range[a]	Virus	Exposure dose[a]	Critical values[b]		MOI[c]	
			Lower	Upper	LLI	ALI
116 - 260	Thai/16	126	105	149	$2x10^{-4}$	$6x10^{-5}$
	Anhui/1	281	249	315	$5x10^{-4}$	$1x10^{-4}$
	Panama/99	138	115	161	$2x10^{-4}$	$7x10^{-5}$
7.2 - 17	Thai/16	16	9	25	$3x10^{-5}$	$8x10^{-6}$
	Anhui/1	18	11	27	$3x10^{-5}$	$9x10^{-6}$
	Panama/99	8	3	14	$1x10^{-5}$	$4x10^{-6}$
1.3 - 1.6	Thai/16	1	0	4	$2x10^{-6}$	$7x10^{-7}$
	Anhui/1	2	0	5	$3x10^{-6}$	$9x10^{-7}$
	Panama/99	1	0	4	$2x10^{-6}$	$7x10^{-7}$

[a]Expressed as PFU/well

[b]Values for a 95% confidence level assuming that presented dose follows a binomial distribution with $N = N_{cham}$ and $p = SA/XA$. The true presented dose for any given well has a 95% chance of falling between these two values.

[c]Multiplicity of infection (MOI) calculated for alveolar or Calu-3 cells exposed to virus, expressed as PFU/cell

Supplementary Experimental Procedures

Derivation of exposure dose formula

The concentration of infectious virus in an aerosol (C_{aer}) can be calculated using the media volume (V_{samp}), concentration (C_{samp}), and flow rate (Q_{samp}) of the sampler as well as the exposure time (t) (equation 1). When the system is used for animal inoculation, presented dose—the dose inhaled by the animal—is estimated by multiplying C_{aer} by the animal's minute volume (MV) and the exposure time (equation 2) (Gustin et al., 2011). Combining equations 1 and 2, and representing the total number of infectious virus particles in the sampler as N_{samp}, allows for the calculation of presented dose based on measured parameters (equation 3). Because the ratio of viral particles in the sampler (N_{samp}) to the total number of aerosolized infectious particles passed through the chamber (N_{cham}) is equal to the ratio between the flow rate of the sampler and the flow rate of the chamber (equation 4), equation 3 can be rewritten in terms of the flow rate and number of infectious viral particles to pass through the entire chamber (equation 5). Multiplying the right side of this equation by time/time shows that the presented dose is equal to the total number of aerosolized particles passing through the chamber multiplied by the ratio of the total volume of air inhaled by the ferret (V_{fer}) to the total volume of air that passed through the chamber during the exposure period (V_{cham}) (equation 6).

In order to account for the fact that we were exposing a two-dimensional, rather than three-dimensional surface to the aerosol, we modified this equation so as to determine the dose of virus contacting the surface of each well by multiplying the total number of aerosolized particles passing through the chamber (N_{cham}) by the ratio of the surface area of the well (SA) to the cross-sectional area (XA) of the chamber. Substituting measured parameters for the N_{cham} term (equation 4) yields equation 7. Exposure dose

was substituted for presented dose as the deposition efficiency was found to be approximately 100%. The concentration in the nebulizer needed to achieve a particular exposure dose can be calculated using equation 8, where *SF* represents the spray factor, the ratio of the concentration of virus in the aerosol to its concentration in the nebulizer, for a given virus stock (Hartings and Roy, 2004).

Equations

1) $C_{aer} = \frac{(C_{samp})(V_{samp})}{(Q_{samp})(t)}$

2) $PD_{fer} = (C_{aer})(MV)(t)$

3) $PD_{fer} = \frac{(N_{samp})(MV)}{Q_{samp}}$

4) $\frac{N_{samp}}{N_{cham}} = \frac{Q_{samp}}{Q_{cham}} \quad N_{samp} = \frac{(Q_{samp})(N_{cham})}{Q_{cham}} \quad N_{cham} = \frac{(N_{samp})(Q_{cham})}{Q_{samp}}$

5) $PD_{fer} = \frac{(\cancel{Q_{samp}})(N_{cham})(MV)}{(\cancel{Q_{samp}})(Q_{cham})}$

6) $PD_{fer} = \frac{(N_{cham})(V_{fer})}{(V_{cham})}$

7) $ED_{cells} = PD_{cells} = \frac{(SA_{well})(N_{samp})(Q_{cham})}{(XA_{cham})(Q_{samp})}$

8) $C_{neb} = \frac{(XA)(ED)}{(SA)(Q_{cham})(SF)(t)}$

Chapter 3: Infection and replication of influenza virus at the ocular surface

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Running title: Influenza virus at the ocular surface

Abstract word count: 248

Text word count (excludes refs, table footnotes, figure legends): 5600

Keywords: Influenza, eye, ocular, tropism, aerosol, tears, tear film, conjunctivitis

Abstract

Though influenza viruses typically cause respiratory tract disease, some viruses, particularly those with an H7 hemagglutinin, have been isolated from the eyes of conjunctivitis cases. Previous work has shown that isolates of multiple subtypes from both ocular and respiratory infections are capable of replication in human *ex vivo* ocular tissues and corneal or conjunctival cell monolayers, leaving the determinants of ocular tropism unclear. In this study, we evaluated the effect of several variables on tropism for ocular cells cultured *in vitro* and examined the potential effect of the tear film on viral infectivity. All viruses tested were able to replicate in primary human corneal epithelial cell monolayers subject to aerosol inoculation. The temperature at which cells were cultured post-inoculation minimally affected infectivity. Replication efficiency, in contrast, was reduced at 33°C relative to 37°C and this effect was slightly greater for the conjunctivitis isolates than the respiratory ones. With the exception of a seasonal H3N2 virus, the subset of viruses studied in multi-layer corneal tissue constructs also replicated productively after either aerosol or liquid inoculation. Human tears significantly inhibited hemagglutination of both ocular and non-ocular isolates, but the effect on viral infectivity was more variable, with tears reducing the infectivity of non-ocular isolates more than ocular isolates. These data suggest that most influenza viruses may be capable of establishing infection if they reach the ocular surface, but that this is more likely for ocular isolates as they are better able to maintain their infectivity in the presence of tears.

Introduction

In contrast to other influenza A viruses, which rarely cause ocular symptoms, H7 subtype viruses have historically shown a unique propensity to infect the eye, typically resulting in conjunctivitis. In fact, prior to 2013, the majority of human infections with H7 viruses presented with conjunctivitis, sometimes accompanied by mild respiratory symptoms, suggesting that the H7 hemagglutinin may be an important determinant of ocular tropism. However, there was a notable absence of conjunctivitis during the first wave of human infections with H7N9 viruses in China, and to our knowledge, there have been no subsequent reports of ocular symptoms associated with any of the over 1500 cases reported to date (1-3). This indicates that the presence of an H7 hemagglutinin may be required for ocular tropism, but is not sufficient to confer this property. At this time, the factors that enable certain H7 viruses to infect and replicate in the eye remain unclear.

Neither receptor binding specificity nor protease susceptibility, two major determinants of influenza viral tropism, can currently explain why such a limited set of viruses appear capable of causing ocular infections given that the eyes are presumably exposed to similar fomites, respiratory droplets, and aerosols as is the respiratory tract. With the exception of highly pathogenic avian strains, influenza viruses have traditionally been thought to be limited to the respiratory and intestinal tracts due to their dependence on host proteases present only in these tissues for hemagglutinin cleavage (4). However, the fact that low pathogenic avian influenza viruses have been associated with conjunctivitis suggests that proteases present in the human eye are capable of performing this function (5, 6). Similarly, the predominance of α2,3-linked sialic acid on the ocular

surface may explain poor susceptibility to human influenza viruses, which preferentially bind α2,6-linked glycans, but not to avian H5 or H9 viruses (7-9)

Previous studies have shown that influenza A viruses of multiple subtypes can replicate in various human ocular cells and *ex vivo* tissues (8, 10-12). This suggests that tropism may be affected by cellular characteristics, such as mucin expression, that differ between cells present in the eye and those cultured in the laboratory. Alternatively, tropism for ocular tissue may not be governed by cellular susceptibility and permissiveness, but by conditions external to cells themselves. We considered both possibilities in this study: in addition to examining how viral replication in corneal epithelial cells was affected by inoculation method, temperature, and culture complexity, we also investigated the interaction between influenza viruses and human tears. Our data ultimately supported the latter hypothesis, suggesting that the ability to retain infectivity during passage through the tear film may determine which viruses are able to establish infection of the eye.

Results

Ocular and non-ocular influenza viruses are capable of replication in primary human corneal epithelial cells

To confirm previous findings in our experimental system, we first evaluated the ability of a panel of five influenza A viruses (shown in Table 1), to replicate in primary human corneal epithelial cells. The panel included representative highly pathogenic avian influenza (HPAI) viruses isolated from ocular swabs of human conjunctivitis cases associated with poultry outbreaks in The Netherlands (NL/230) and Mexico (Mex/7218)

in 2003 and 2012, respectively. Both will hereafter be described as ocular or ocular tropic. For comparison, we used a virus from one of the early H7N9 cases in China (Anh/1), an HPAI H5N1 virus (Thai/16), and a seasonal H3N2 virus (Pan/99). These viruses were isolated from the respiratory tract of human cases that displayed no ocular symptoms and are considered respiratory, or non-ocular tropic. In accordance with the literature, all viruses tested were able to replicate productively, reaching titers of at least 10^4 PFU/mL following inoculation at an MOI of 0.01 (Fig. 1).

Inoculation method and culture temperature do not alter tropism for corneal cells *in vitro*

Though some persons experiencing influenza-associated conjunctivitis report trauma to the eye (6, 13), for most, the only known exposure is contact with infected poultry, often during culling operations, for which eye protection is recommended, but not necessarily worn (14, 15). This suggests that ocular infection usually occurs either from workers touching their eyes with contaminated hands or from virus traveling through the air. We hypothesized that ocular viruses might be better able than others to infect ocular cells via aerosol, or might replicate more efficiently at the sub-37°C temperature of the human ocular surface. To determine if this was the case, we exposed corneal cell monolayers to four different aerosol doses of the viruses examined previously. After simultaneous inoculation, half of the culture wells were incubated at 37°C and the other half at 33°C.

As with traditional liquid-based inoculation, all viruses were able to replicate productively after aerosol inoculation. This was true even for very low doses of virus. For cells cultured at 37°C, 50% infectious doses (ID_{50}s) were $\leq$ 10 PFU (Fig 2). At 33°C,

ID_{50}s were similar, except in the case of NL/230 virus, which had an ID_{50} of 169 PFU at 33°C compared to 10 PFU at 37°C. Consistent with data in respiratory cells (16), temperature had little effect on the growth of the seasonal virus Pan/99 whereas growth of the avian viruses was attenuated at the lower temperature. The effect of temperature on curve shape was similar across inoculation doses, but differences in peak titer were greater at lower exposure doses, possibly because the low dosage delayed replication at both temperatures such that titers had not plateaued by 96 hours post-infection.

In order to confirm that our findings were not specific to individual virus strains, but could be applied to groups of similar isolates, we expanded our panel of viruses (Table 1) to include an additional HPAI H7N7 ocular virus, Italy/3, isolated from the eye of a poultry worker with conjunctivitis, and three respiratory viruses: the early pandemic H1N1 isolate Mex/4482, one from a single human H7N2 respiratory case that occurred in New York in 2003 (NY/107), and an additional human HPAI H5N1 isolate (Bang/5487). NL/219 virus, which was isolated from the lone fatal human case associated with the same 2003 Netherlands conjunctivitis outbreak as NL/230 virus and differs from NL/230 by 15 amino acids, including position 627 of PB2 (lysine in NL/219 vs glutamic acid in NL/230), was also included. Symptoms associated with this case were exclusively respiratory and the virus was obtained from a post-mortem lung specimen (17). A single, low inoculation dose (target dose ~10 PFU) was used. Inoculations occurred via the aerosol route and all cells were cultured at 37°C post-inoculation. Again, all viruses tested were able to establish infection and replicate, ultimately reaching titers of at least 10^5 PFU/mL (Fig. 3). Replication curves for Pan/99 (seasonal H3N2) and Mex/4482 (pandemic H1N1) viruses were nearly identical, with a steady increase in viral titer

between 24 and 96 hours after infection. The two respiratory H7 subtype viruses showed similar growth kinetics, though the titers for Anh/1 virus were approximately 10 times higher than those for NY/107 virus. In contrast, the two H5N1 viruses grew more rapidly early after inoculation, with titers plateauing after 48 hours. Growth kinetics of the three ocular viruses tested varied somewhat, differing by about three $\log_{10}$ PFU at the 24 hour timepoint, but all peaked at approximately the same titer ($10^{5.5}$ PFU/mL). The growth of NL/219 virus was similar to that of the H5N1 viruses, with a maximum titer 100-fold higher than that of the H7 ocular isolates. In summary, neither inoculation method nor culture temperature restricted the ability of respiratory isolates to productively infect human corneal epithelial cells.

Viral infection of corneal tissue constructs

While cryopreserved cells grown in monolayers are a useful and flexible research tool, there are many features of the ocular surface that are potentially relevant to viral infection, but are not recreated by typical culture methods. We next examined commercially available corneal tissue constructs (EpiCorneal, Mattek) to determine whether these might provide an enhanced *in vitro* model of influenza ocular infection. The tissue constructs comprise a stratified epithelium with approximately five layers of cells and are grown at air-liquid interface. While low transepithelial resistance indicated that the corneal cell monolayers described above lacked tight junctions, these were present in the apical layer of the constructs, as shown by positive ZO-1 staining (Fig. S1). Additionally, expression of the major tethered corneal mucins, MUC1, MUC4, and MUC16, was greater in the constructs than in the cell monolayers (Fig. 4). Staining with

SNA and MAA-I lectins indicated that both α2,6 and α2,3-linked sialic acids were present on the apical surface of both monolayers and constructs, though, consistent with human *ex-vivo* tissue staining of the conjunctiva, α2,3 linkages were more prevalent (Fig. 5) (7). In contrast to the respiratory cell line Calu-3, in which the two lectins stain distinct cells, many of the corneal cells were bound by both lectins, suggesting coexpression of the two types of sialic acid receptors.

Though we had found that a variety of viruses could infect and replicate in corneal cell monolayers after aerosol inoculation, we suspected that the more extensive mucin layer and greater differentiation of cells in the constructs might inhibit infection by some influenza viruses more than others. We therefore exposed constructs to one of three different doses of aerosolized NL/230, Anh/1, Thai/16, or Pan/99 virus, or to a 0.01 MOI liquid inoculum. Infection and replication were limited at the lowest (≤5 PFU) aerosol dose, with only NL/230 virus reaching titers above 100 PFU, and only in 2/3 replicates (Fig. 6). At higher doses viral replication appeared to be productive for NL/230, Anh/1, and Thai/16 viruses, with titers increasing over time, particularly in the first 48 hours. Limited production of Pan/99 virus was seen in the liquid-inoculated wells whereas the aerosol-inoculated ones either did not increase in titer or dropped to undetectable levels at 48 hours post-infection.

Given that both aerosol and liquid inocula reliably infected cells, liquid-based inoculation was used for all further experiments in order to reduce variability in multiplicity of infection. First, we generated 0.01 MOI replication curves for four additional viruses in the corneal constructs. Mex/7218 and NL/219 viruses replicated most efficiently, with the growth curve of NL/219 virus resembling that of Thai/16,

although, unlike Thai/16, it plateaued by 48 hours (Fig 7A). Unlike Anh/1, an additional H7 respiratory virus (NY/107) replicated relatively poorly, with titers similar to those of the seasonal Pan/99 virus. Mex/4482, a pandemic H1N1 virus, grew slightly better, with supernatant concentrations at 96 hours post-infection approximately ten times those of Pan/99 and NY/107 viruses. In order to evaluate the spread of infection within the construct, we fluorescently stained constructs infected with each virus at 96 hours post-infection using an antibody against the viral nucleoprotein (NP). We had anticipated that cells deeper in the stratified epithelium might become infected as the viruses spread, but instead found that viral nucleoprotein was generally limited to the apical layer of cells (Fig. 7B).

To compare the ability of different influenza viruses to initiate infection, we inoculated the constructs at an MOI of 2, fixed the cells 8 hours later, and fluorescently stained the viral NP (Fig. 8A). We quantified the level of infection by both counting cells and measuring the mean fluorescence activity per 10x field. We found more NP+ cells and more intense cytoplasmic staining around positive nuclei in constructs inoculated with an ocular H7 virus than in those inoculated with a respiratory H7 virus (Fig. 8B), but neither the difference in the number of NP+ nuclei nor in mean fluorescence intensity was statistically significant, regardless of whether NL/219 virus was included with the two other ocular viruses or excluded from the analysis. Differences in infectivity were much more drastic when we compared the seasonal H3N2 virus (Pan/99) and pandemic H1N1 (Mex/4482) isolates (Fig. 8C). An average of only 6.3 nuclei per field were positive for Pan/99 as compared to 73 for Mex/4482 virus (p=0.013). As with the non-ocular H7 viruses, Pan/99 staining was concentrated, presumably in the nucleus, whereas

the Mex/4482 staining pattern was closer to that seen with the ocular H7s. These data indicate that although poor ability to infect and replicate within the eye may account for the infrequency with which conjunctivitis is observed in association with seasonal influenza virus infection, it does not explain why only certain avian viruses tend to cause ocular symptoms.

Ocular viruses are less inhibited by human tears than are non-ocular viruses

Given the ability of so many different viruses unassociated with ocular infection to replicate in corneal epithelial cells, the apparent rarity with which they do so in humans suggested to us that infectious virions may not even reach the cell surface. Specifically, we suspected that non-ocular tropic viruses may be more susceptible than ocular viruses to inhibition by the tear film, a thin (7-8μm thick) layer comprised of lipid, aqueous, and mucus components known to play an important role in protecting the eye from microbial pathogens (19, 20). Because direct sampling of the tear film yields very minimal volume (tear volume in the eye is approximately 7μL, only about 2μL of which can be collected without stimulating tearing (19)) we elected to use induced tears for our studies. Human tears had relatively high hemagglutination inhibition titers (GMT>300) against all viruses tested except NL/230. Overall, titers for NL/219 as well as the two ocular viruses were lower than for the non-ocular viruses (Table 2).

To evaluate the effect of tears on viral viability, we employed a plaque reduction assay. Virus was mixed with an equal volume of either PBS or diluted tears, incubated for one hour at room temperature, then plated on MDCK cells for standard plaque assay. Viral infectivity remaining post-incubation was calculated by dividing the number of

plaques on each tear-treated well by the number on the corresponding PBS well. All of the viruses tested were significantly inhibited by tears diluted 20-fold (Fig. 9A, black bars). As a group, the ocular viruses were less inhibited than were the non-ocular ones ($p<0.001$ whether or not NL/219 virus was included in the ocular group), though the percentage of the H5N1 virus, Thai/16, which remained infectious after incubation with the tears was within the range of the ocular viruses. Tears from a second set of donors were less inhibitory overall, but the difference between ocular and respiratory viruses was maintained (data not shown). A dose-response relationship was evident when tears were further diluted, with higher percentages of virions retaining infectivity when mixed with more diluted tear samples, but this relationship was not identical across viruses, suggesting that different viruses might be primarily inhibited by tear components present in differing concentrations.

In order to investigate what component or components of tears were responsible for the reduction in viral infectivity that we observed, we first tested two antimicrobial proteins present in large quantities in tears: lactoferrin and lysozyme (21). Each was substituted for tears in the plaque reduction assay at a concentration equivalent to that expected to be present in the highest concentration of tears used previously (a 1:20 dilution). The viability of all viruses except NL/219 remained high (>75% of control) after incubation with both proteins, and no difference between ocular and non-ocular viruses was evident (Fig. S2). NL/219 virus was uniquely affected, with only 53% and 39% infectivity retained after treatment with lactoferrin and lysozyme, respectively.

To evaluate the extent to which the inhibition we observed was due to the binding of virus to sialic acid present in the tear fluid, we desialylated the tears with exogenous

bacterial neuraminidase prior to incubation with virus. Desialylation was confirmed by reduction of HI titer to <10. This approach required incubating the tears-neuraminidase mixture at 37°C (to facilitate neuraminidase activity), then heating to a higher temperature to inactivate the neuraminidase so that it would not prevent infection by removing the sialic acid receptors from the surface of the MDCK cells used in the plaque reduction assay. However, we found that when mixed with virus after putative inactivation at the standard conditions of 56°C for 30 minutes, neuraminidase alone (in PBS) decreased plaque number relative to that seen with PBS only. Increasing the inactivation temperature to 65°C reduced, but did not eliminate this effect, the magnitude of which differed by virus (Fig. S3). We therefore used two controls for each experimental sample: one contained tears, but no neuraminidase (to control for the impact of heating on the inhibitory properties of tears) and the other contained neuraminidase, but no tears (to control for the residual effect of the neuraminidase). Both were subjected to the experimental conditions (37°C for one hour followed by 65°C for 30 minutes) and the combined effect of the two was estimated by adding the individual effects. We then compared the number of infectious particles remaining after incubation with desialylated tears to the number expected from a single, theoretical control reaction (Fig. 9B). The difference between the two, representative of the percentage of PFU inhibited by sialic acid, was greater for ocular (69%) than non-ocular (52%, $p<0.001$) viruses. In conducting these experiments, we found that heating tears (37°C for one hour followed by 30 minutes at 65°C) reduced their inhibitory activity. This was particularly true of the respiratory viruses, for which the difference in post-incubation viral viability between heat-treated and non-heat-treated tears averaged 29% as compared to 7% for the ocular

viruses (Fig. 9C, $p < 0.001$, ocular group includes NL/219). These data demonstrate that human tears are highly effective in blocking influenza viral infection and suggest that multiple tear components are responsible for this effect.

Discussion

Despite previous studies conducted *in vivo*, *ex vivo*, and *in vitro*, the unique characteristics of influenza viruses associated with ocular infection remain unclear. Whether only a minority of influenza viruses are capable of infecting and replicating in the human eye, or whether ocular involvement is routine, but only triggers sufficient inflammation to cause symptoms in select cases, remains an open question as the eye is not usually sampled in patients with typical influenza-like illness. Previous studies have demonstrated the ability of non-conjunctivitis-associated viruses to replicate in primary human ocular cells and *ex vivo* tissues. While this work suggests that cells present on the ocular surface are both susceptible and permissive to infection, it does not necessarily indicate that these viruses are ocular-tropic in humans, where conditions may differ from those used in the laboratory. In this study, we examined the influence of culture temperature, inoculation method, and membrane-associated mucins on the ability of a variety of influenza viruses to infect and replicate within human corneal epithelial cells, and investigated the interaction between different influenza viruses and the tear film.

Though ocular aerosol challenge has been used in animals, to our knowledge, this inoculation method has not been used with human ocular cells or tissues. We previously found that primary human alveolar epithelial cells were less susceptible to infection with certain viruses when inoculated via aerosol rather than by more traditional methods (16).

To determine whether a similar phenomenon might explain why both seasonal and avian respiratory viruses, previously shown to productively infect multiple ocular cell types (7, 8, 10, 11, 22), rarely establish ocular infection in humans, we inoculated corneal epithelial cells by the aerosol route. All viruses examined proved capable of infecting primary human corneal epithelial cell monolayers after aerosol delivery, suggesting that this is a plausible route of infection, but not indicating that aerosol exposure can account for differences in ocular tropism.

In contrast to core body temperature, the surface of the eye, like the upper respiratory tract, is approximately 33°C (23, 24). While the relationship between the efficiency of replication in respiratory cells at 33°C and virus transmission has been extensively studied, minimal work has been performed on the role of temperature in ocular infection. Some studies have used a culture temperature of 33°C and others 37°C (7, 8, 10, 11), but a comparison between the two temperatures has not been made. Though ocular isolates, including the two used in this study, typically do not have markers (eg. PB2 627K or 701N) associated with enhanced replication efficiency at lower temperatures, and replicate poorly at 33°C compared to 37°C in respiratory and MDCK cells, we nonetheless tested the hypothesis that ocular influenza viruses might either infect or replicate better than non-ocular viruses at 33°C, as this would be consistent with their ability to establish infection at the ocular surface. However, we found that the effect of culture temperature on viral infectivity and replication in corneal cells was similar to that observed in other cell types.

Membrane associated mucins serve a critical barrier function in the eye, preventing pathogens from reaching the immune-privileged cornea (18, 25). These

heavily glycosylated proteins are thought to entrap influenza virus particularly well as the viral hemagglutinin binds efficiently to their abundant sialic acid residues (26). Because the primary cell monolayers used in our initial experiments expressed these mucins at very low levels, we conducted a parallel set of experiments using corneal tissue constructs, which are cultured at air-liquid interface and express high levels of these mucins at the apical surface. While mucins have been hypothesized to be important determinants of ocular tropism (27), we did not find evidence that ocular-tropic viruses had an advantage in infection or replication in their presence, regardless of inoculation method.

The use of multi-layer tissue constructs also allowed us to examine viral spread within the stratified corneal epithelium. We predicted that ocular influenza viruses might be better able than others to spread, either directly between cells or by diffusion into damaged areas, and would therefore infect deeper layers of the epithelium. In fact, both ocular and respiratory viruses were confined to the apical portion of the constructs. The presence of some NP in the basolateral layer of one construct (Fig. 8B, Anh/1 virus) demonstrates that the anti-NP antibody was able to penetrate deep into the tissue construct, indicating that the lack of positive staining below the apical layer for other viruses reflects the absence of virus and not a limitation of the staining process itself. This limited distribution of viral NP is consistent with patient symptoms. Though influenza ocular infections are typically described as conjunctivitis, symptoms present in some cases, such as pain and sensitivity to light (6, 28), suggest corneal involvement (29, 30). The inability of virus to spread beyond the superficial squamous epithelial layer likely explains why changes in vision, which are common features of herpes keratitis, and

sometimes adenoviral keratoconjuncitivis, have not been reported (29, 31). Alternatively, infection may be limited to the conjunctiva and symptoms result from the spread of inflammation to the cornea. Further investigation of the extent of differentiation of cells in tissue constructs and of factors that limit spread deeper into the corneal epithelium is warranted.

We found that human tears inhibited viral hemagglutination and infection, even when diluted 20-fold or more. As a group, the ocular influenza viruses retained more infectivity than did their non-ocular counterparts when incubated with tears. Interestingly, the non-ocular H7 viruses were particularly susceptible to inhibition, suggesting that non-hemagglutinin viral proteins, such as the neuraminidase, affect the ability of viruses to avoid inactivation before reaching the host cell surface. The tear film includes a wide variety of antimicrobial factors, including secretory immunoglobulin A (IgA), lipocalin, lactoferrin, lysozyme, and mucins. IgA undoubtedly plays an important role in the protection of the eye from numerous pathogens, possibly including seasonal influenza virus, but cannot account for the infrequency of ocular infection by swine and avian-origin influenza viruses as antibodies to these viruses are rare in humans. Additionally, IgA is present in cried tears at much lower levels than in basal tears, making it difficult to study without tear specimens obtained by capillary (32). We did not examine lipocalin individually as neither its ability to bind microbial siderophores nor its DNase activity made it a likely candidate for inhibition of an RNA virus (33, 34). In contrast, antiviral effects of lactoferrin have been documented for numerous viruses (35, 36). In the case of influenza virus, Ammendolia and colleagues found that when incubated with multiple viral subtypes prior to cellular inoculation, even very low concentrations of bovine

lactoferrin were able to prevent cytopathic effects by binding to the first 18 amino acids of the fusion peptide of the hemagglutinin (37). Although bovine lactoferrin is 69% identical to human lactoferrin at the amino acid level and the two structures are similar (38), it differs from the human protein by nearly 50% across the three loops thought to bind influenza hemagglutinin. Our use of human lactoferrin thus likely explains why we observed such a limited effect on viral viability. Infectivity of NL/219 virus, which was substantially reduced by lactoferrin, was probably affected by a different mechanism as this virus is identical across the relevant portion of the fusion peptide to the other, less-affected, H7 viruses tested. Known for its ability to cleave the peptidoglycan of the bacterial cell wall, lysozyme also reduces virus production by HIV-infected cells, and, albeit when denatured, inactivates murine and human noroviruses (39, 40). Its impact on the influenza viruses tested in this study was similar to that of lactoferrin, with only a minimal effect on all viruses tested except for NL/219.

Mucins present in the tear film include both membrane associated mucins that have been proteolytically cleaved or have broken away from the cell surface and the secreted gel-forming mucin MUC5AC, which is produced by the conjunctival, but not the corneal, epithelium (41, 42). As noted earlier, the many glycans present on ocular mucins can serve as decoy receptors for sialic-acid (SA) binding pathogens, interfering with attachment to the ocular surface. In hemagglutination inhibition assays, ocular influenza viruses were less inhibited by tears (lower HI titers) than were non-ocular viruses. The fact that hemagglutination inhibition activity was removed by treatment with exogenous neuraminidase strongly suggests that this inhibition was due to SA. However, the plaque reduction assay yielded the opposite result, with the ocular viruses showing greater

infectivity differences between neuraminidase treated and untreated tears. This indicates that binding does not necessarily prevent infection, possibly because the non-ocular viruses have higher neuraminidase activity relative to hemagglutinin affinity, making them better able to free themselves from mucins and go on to initiate infection. Alternatively, the effects of inactivated neuraminidase and of tears are not additive, as we assumed, resulting in an underestimation of the number of plaques on control wells and thus an overestimation, particularly in the case of viruses more affected by the inactivated exogenous neuraminidase, of the infectivity difference between neuraminidase-treated tears and control. In any case, the data do not necessarily suggest, as might have been expected, that viruses with a preference for either α2,6 or α2,3-linked SA receptors are more inhibited by the SA in tears: in the hemagglutination inhibition assay, titers for Pan/99 virus, which binds α2,6-linked receptors, were closer to those of the α2,3-binding ocular viruses than were the titers for Thai/16 virus, a member of a clade which strongly favors α2,3-linked SA (43-45). In the plaque reduction assay, Thai/16 virus behaved more similarly to the ocular viruses than did Pan/99. However, NY/107 and Anh/1 viruses, which prefer α2,3 linked SA, but have limited binding to α2,6-linked SA, did not display an intermediate phenotype but were more inhibited than any of the other viruses (43, 46, 47). The fact that the difference between viral viability retained after incubation with heated and un-heated tears was greater for ocular tropic than respiratory tropic viruses suggests the presence of an inhibitory, heat-labile, component of tears to which ocular influenza viruses are more resistant than those which do not display this tropism in humans. The identity of this component remains unclear; with approximately 500 proteins present in human tears, there are many possible candidates (48).

Although influenza-associated conjunctivitis occurs quite rarely, the potential for the eye to serve as a point of entry and possible human adaptation for viruses which are poorly suited to establishing infection in the respiratory tract represents a cause for public health concern. Knowledge of the factors which permit or restrict ocular infection is key to assessing the significance of this risk. Our findings reinforce the importance of examining the extracellular environment as well as the intracellular one in studies of host-virus interaction.

Materials and Methods

Viruses

Viruses used in this study are listed in Table 1. Virus stocks were generated either in Madin-Darby canine kidney (MDCK) cells (Mex/4482, Bang/5487) or the allantoic cavity of 10-day-old embryonated hens' eggs (all other viruses), as described previously (49, 50). HPAI and LPAI viruses were handled under biosafety level 3 containment, including enhancements as required by the U.S. Department of Agriculture and the Federal Select Agent Program (51).

Cells

Human primary corneal epithelial cells (cryopreserved at passage 1) were obtained from ATCC and grown according to the manufacturer's instructions. Cells were subcultured and grown to confluence on 24mm membrane inserts (Transwells, Corning). EpiCorneal tissue constructs (MatTek Corporation) were unpacked immediately upon receipt and pre-equilibrated overnight before use the next morning. Constructs were

maintained at air-liquid interface per manufacturer directions. Media for both corneal monolayers and tissue constructs was serum-free. Human bronchial epithelial cells (Calu-3, ATCC) were propagated as described previously (52).

Cell infections

MOIs for corneal constructs were calculated based on the midpoint (75,000) of the manufacturer's estimate of 50,000 to 100,000 cells on the apical surface (MatTek, personal communication). Aerosol inoculations were performed as described previously (16). Briefly, after removal of apical media, if present, cells were placed into an aerosol exposure chamber for a 15-minute exposure to aerosolized virus, followed by five minutes of room air to clear viral aerosols from the system. For liquid inoculations, apical media was removed from cell monolayers and 150μL (constructs) or 300μL (monolayers) media containing the specified amount of virus was incubated atop the apical surface at 37°C for one hour, after which inoculum was removed. Cell monolayers were washed with PBS, and apical media of cell monolayers was restored, with the addition of 1 μg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich). Trypsin was not added to the tissue constructs, for which apical media was not present. At indicated time-points, 200μL of supernatant (incubated atop tissue constructs for 20 minutes before collection) was collected and an equal volume of fresh, trypsin-containing medium was added to the monolayers. Samples were frozen at -80°C until titration by standard plaque assay in MDCK cells.

Hemagglutination inhibition and infectivity reduction assays

Human tears, pooled from healthy donors were obtained from Lee BioSolutions and, except where noted, a single lot was used for all experiments. Tears were induced reflexively or through emotions and collected from the face. Turkey red blood cells (0.5%) were used for all hemagglutination inhibition assays. Since we were specifically interested in the inhibitory effect of sialic acid hemagglutination, tears were not pretreated with receptor destroying enzyme. Similarly, adsorption of non-specific agglutinins was not performed as there was no evidence of agglutination when tears alone were mixed with red blood cells.

For plaque reduction assays, tears were diluted 10-fold, 20-fold, or 40-fold in PBS, mixed with an equal volume of virus diluted to between approximately 60 and 200 PFU/100μL, and incubated at room temperature for one hour, after which the mixture was used to inoculate MDCK cells for a standard plaque assay. Virus mixed with PBS, also incubated for one hour at room temperature, was used for control wells. For each virus, plaque numbers were summed across three independent assays and the percentage of infectivity remaining was calculated by dividing the total number of plaques in experimental wells by the total number in control wells. Sialidase pretreatment was performed by adding *V. cholera* neuraminidase (Sigma-Aldrich) to tears (experimental wells) or PBS (control wells) yielding a final concentration of 0.02 U/mL and incubated at 37°C for one hour. This treatment was confirmed to reduce the hemagglutination inhibition titer of tears to <10, indicating that sialic acids were successfully removed. For this set of experiments only, PBS with calcium and magnesium was used as a control and as a diluent for the tears because calcium is a required cofactor for *V. cholera* neuraminidase. Lyophilized human milk lactoferrin (Athens Research and Technology)

and recombinant human lysozyme (Sigma-Aldrich) were resuspended in PBS and used at a final concentration of 0.15 mg/mL, equivalent to that expected to be present in a ten-fold dilution of human tears (21, 53-56). Count data from the plaque reduction assay were post-stratified using the R survey package (57, 58) such that each virus contributed equally to the result in spite of differences between viruses in plaque number on control plates. Comparisons were made by chi square test. Binomial confidence intervals for individual viruses were calculated using the binom package (59).

Immunofluorescence

Cells were washed with PBS and fixed using 3% paraformaldehyde. Lectin co-staining was performed using fluorescein-conjugated *Sambucus nigra* lectin (SNA) and biotinylated *Maackia amurensis* lectin I (MAA-I) plus Avidin D-conjugated rhodamine (Vector Laboratories). All other staining was conducted using the primary antibodies listed in Table S2 and an Alexa Fluor 488 or 549-conjugated secondary antibody. Cells were mounted with DAPI and imaged with an LSM710 Zeiss confocal microscope.

Acknowledgements

The authors wish to thank the WHO Global Influenza Surveillance and Response System (GISRS), Erasmus Medical Center, China CDC, the Thai National Institute of Health, the Instituto de Diagnóstico y Referencia Epidemiológicos (Mexico), the Instituto Superiore di Sanità, (Italy), and the Medical Research Council National Institute for Medical Research (United Kingdom) for providing viral isolates. H.M.C. is supported by the Oak Ridge Institute for Science and Education. The findings and conclusions in this

report are those of the authors and do not necessarily reflect the views of the funding agency.

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Tables

Table 1: Influenza viruses used in this study

Virus	Abbreviation	Subtype	Presentation in human case	Reference
A/Panama/2007/1999	Pan/99	H3N2	Seasonal infection	(60)
A/Mexico/4482/2009	Mex/4482	Pandemic H1N1	Severe respiratory infection	(49)
A/New York/107/2003	NY/107	LPAI H7N2	Fatal respiratory infection	(61, 62)
A/Netherlands/219/2003	NL/219	HPAI H7N7	Fatal respiratory infection	(17, 28)
A/Netherlands/230/2003	NL/230	HPAI H7N7	Conjunctivitis	(17, 28)
A/Mexico/InDRE7218/2012	Mex/7218	HPAI H7N3	Conjunctivitis	(63)
A/Anhui/1/2013	Anh/1	LPAI H7N9	Fatal respiratory infection	(64)
A/Italy/3/2013	Italy/3	HPAI H7N7	Conjunctivitis	(65)
A/Thailand/16/2004	Thai/16	HPAI H5N1	Fatal respiratory infection	(50)
A/Bangladesh/5487/2011	Bang/5487	HPAI H5N1	Non-fatal respiratory infection	(66)

Table 2 Heagglutination inhibition by human tears

Virus	GMT[a]
Pan/99	403
Thai/16	640
Anh/1	1016
NY/107	806
NL/219	320
NL/230	10
Mex/7218	403
Mock[b]	<10

[a]Geometric mean titer (n=3).

[b]Normal allantoic fluid

Figures

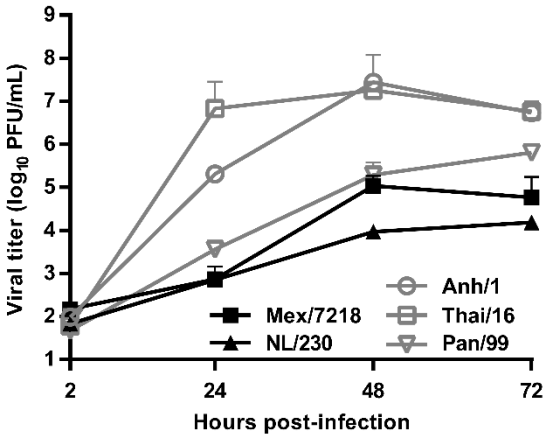

Figure 1. Influenza virus replication in primary human corneal cell monolayers after liquid inoculation. Cells were infected at an MOI of 0.01 with ocular (shown in black) and non-ocular (shown in gray) viruses. Supernatants collected at the indicated times were titered by plaque assay. Each point represents the mean of three replicates, with error bars indicating standard deviation.

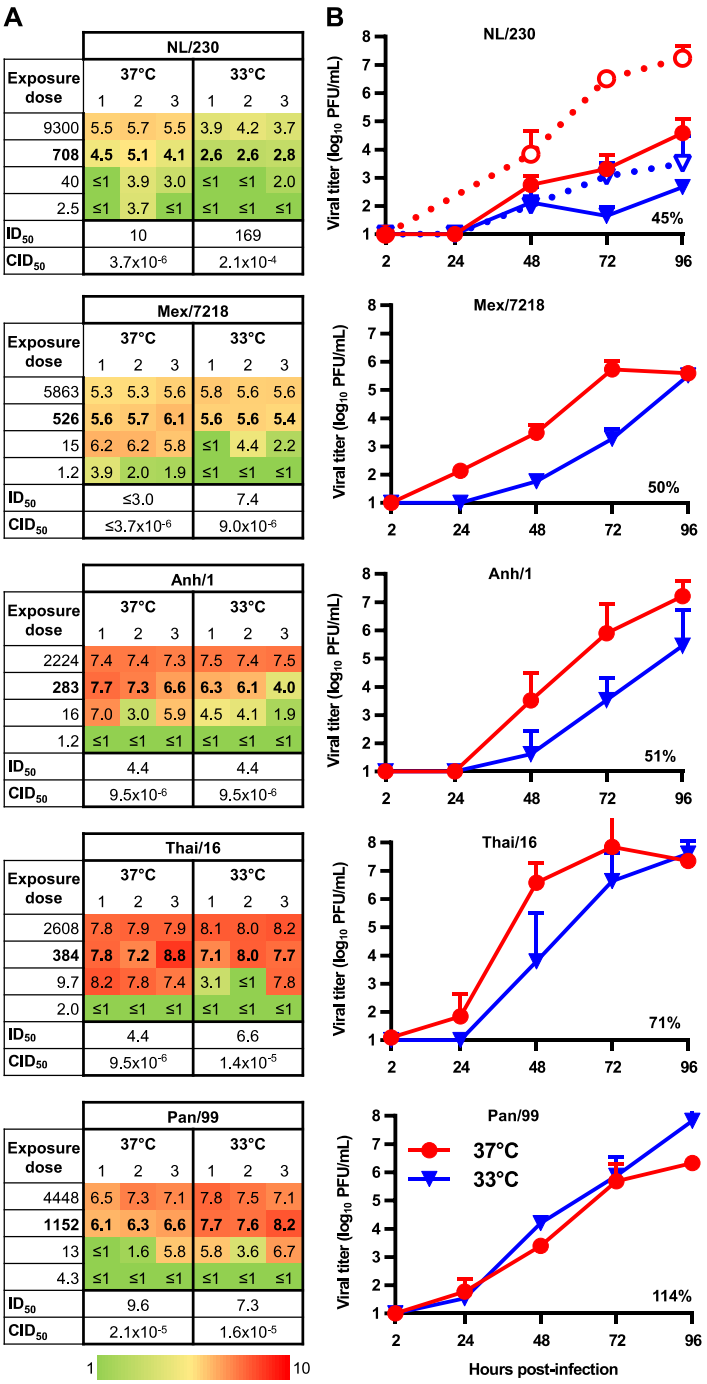

Figure 2. Infectivity and replication in primary human corneal cell monolayers after aerosol inoculation. A) Peak viral titer (log_{10}PFU/mL, limit of detection = 1) for each well exposed to the dose indicated (PFU). CID_{50} is given in PFU/cell and represents the MOI associated with the ID_{50}. B) Replication curves for the bolded rows in A. Dotted

lines and open symbols indicate repeat of experiment with cells from a second donor. Mean + SD is shown. Numbers in lower right of each graph indicate the area under the 33°C replication curve as a percentage of the area under the 37°C curve.

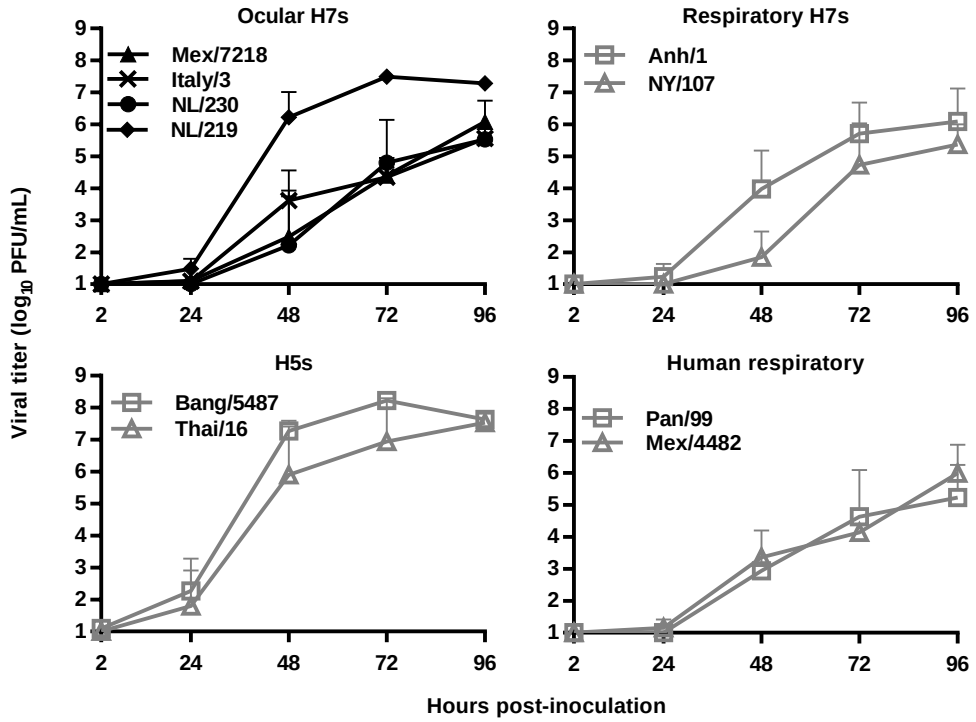

Figure 3. Replication kinetics in primary human corneal cell monolayers infected by low-dose aerosolized virus. NL/230 virus infected only 2/3 wells and the mean of the two infected wells is shown. For all other viruses, each point represents the mean of three independent wells + SD. Exact exposure doses are listed in Table S1. Limit of detection = 1 $\log_{10}$PFU/mL.

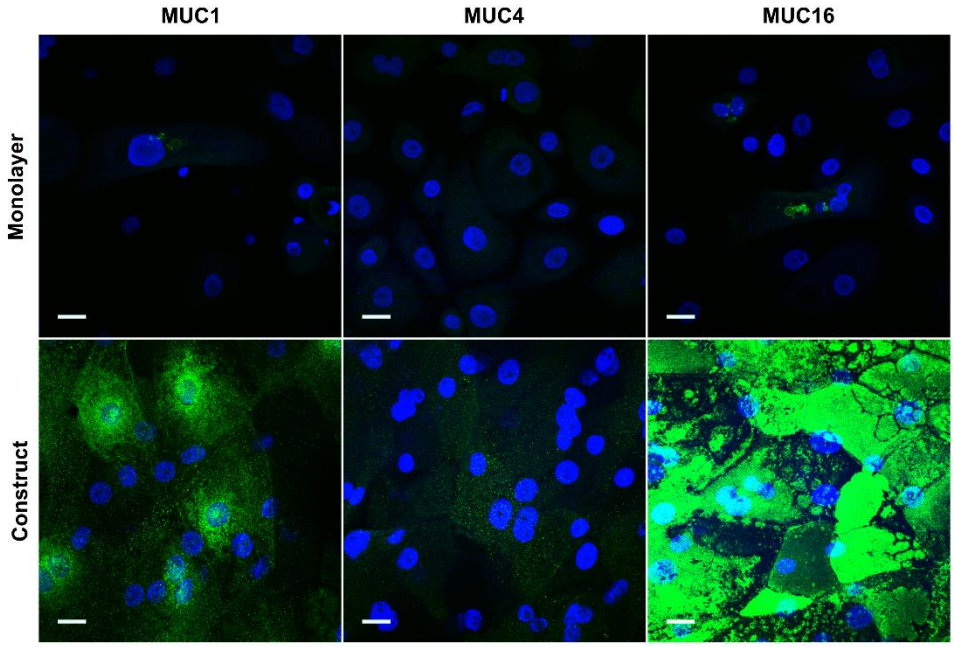

Figure 4. Mucin expression at apical surface of primary corneal cell monolayers and corneal tissue constructs. Uninfected constructs and monolayers were fixed and stained with DAPI (blue) and monoclonal antibodies against MUC1, MUC4, or MUC16 (green). Images are maximum intensity projections across 8.28 μm of the z-axis. Scale bar = 20μm.

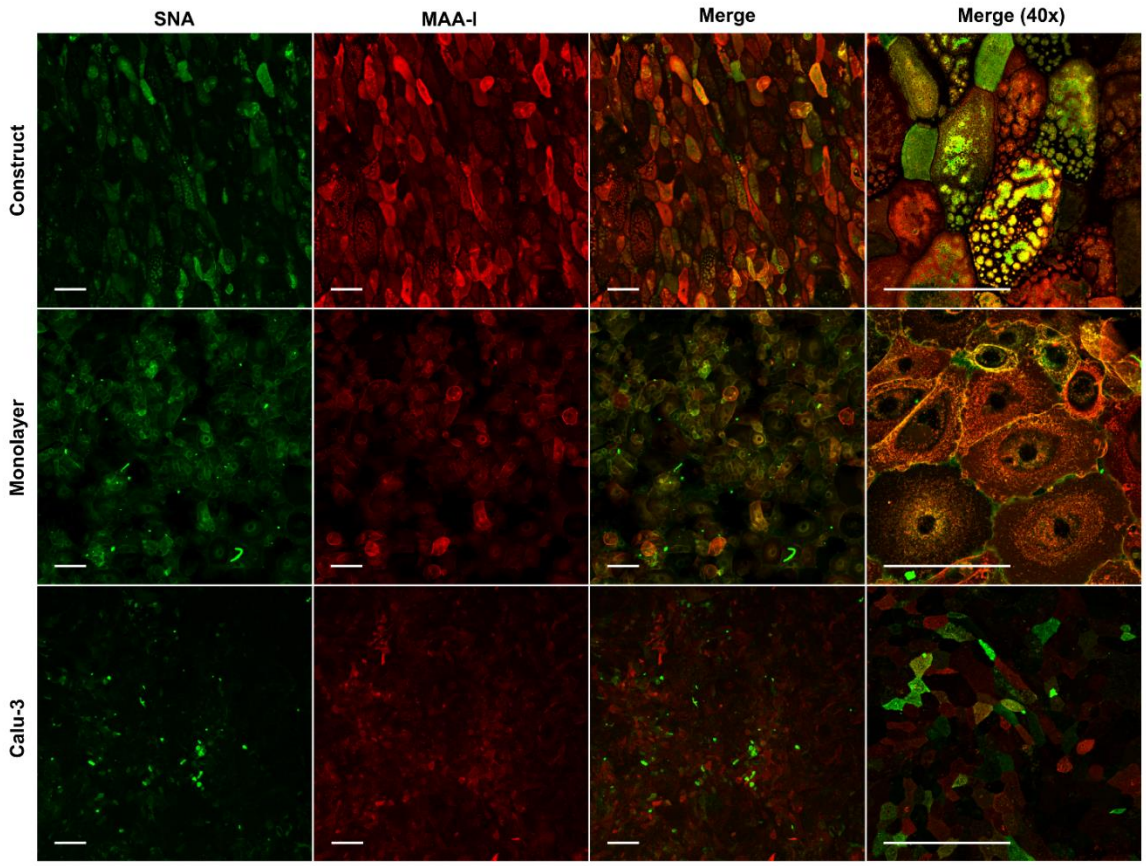

Figure 5. α2,6 and α2,3-linked sialic acid expression on corneal cell monolayers and tissue constructs. The bronchial epithelial cell line, Calu-3, is included for comparison. Cells were stained for α2,6-linked sialic acid using SNA lectin (green) and for α2,3-linked sialic acid using MAA-I (red). Images are maximum intensity projections. Scale bar = 100 μm

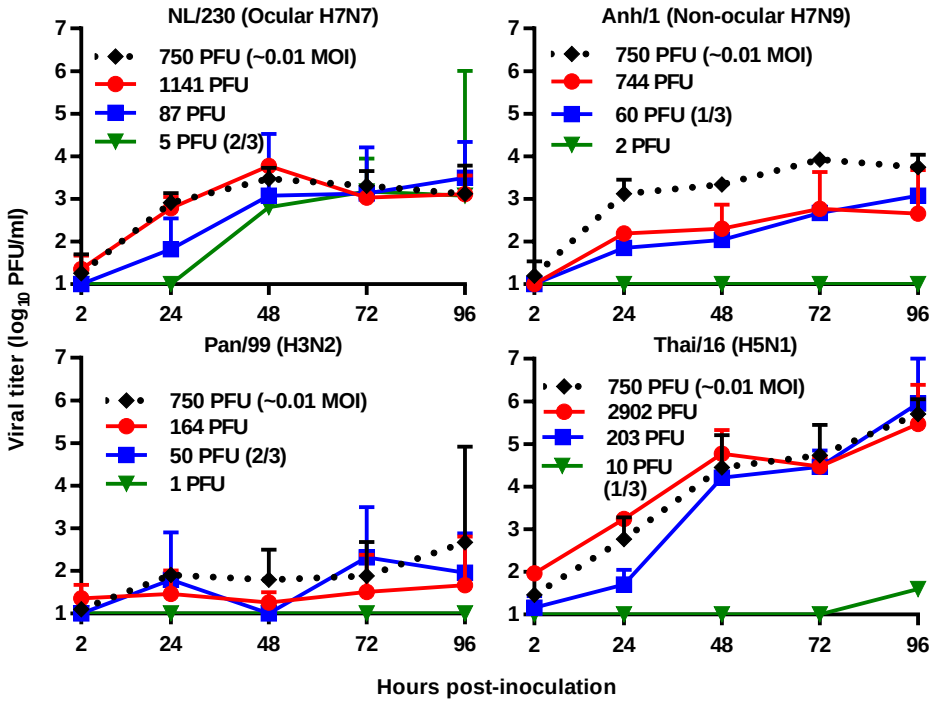

Figure 6. Replication kinetics in corneal tissue constructs. Constructs were exposed with the indicated amount of virus via liquid (dotted line) or aerosol (solid lines) inoculum. The apical surface of each construct was washed with 200µL media at the indicated times post-infection and viral titer determined by standard plaque assay. Mean ± S.D. (n=3) is shown. Limit of detection=1 $\log_{10}$PFU/mL.

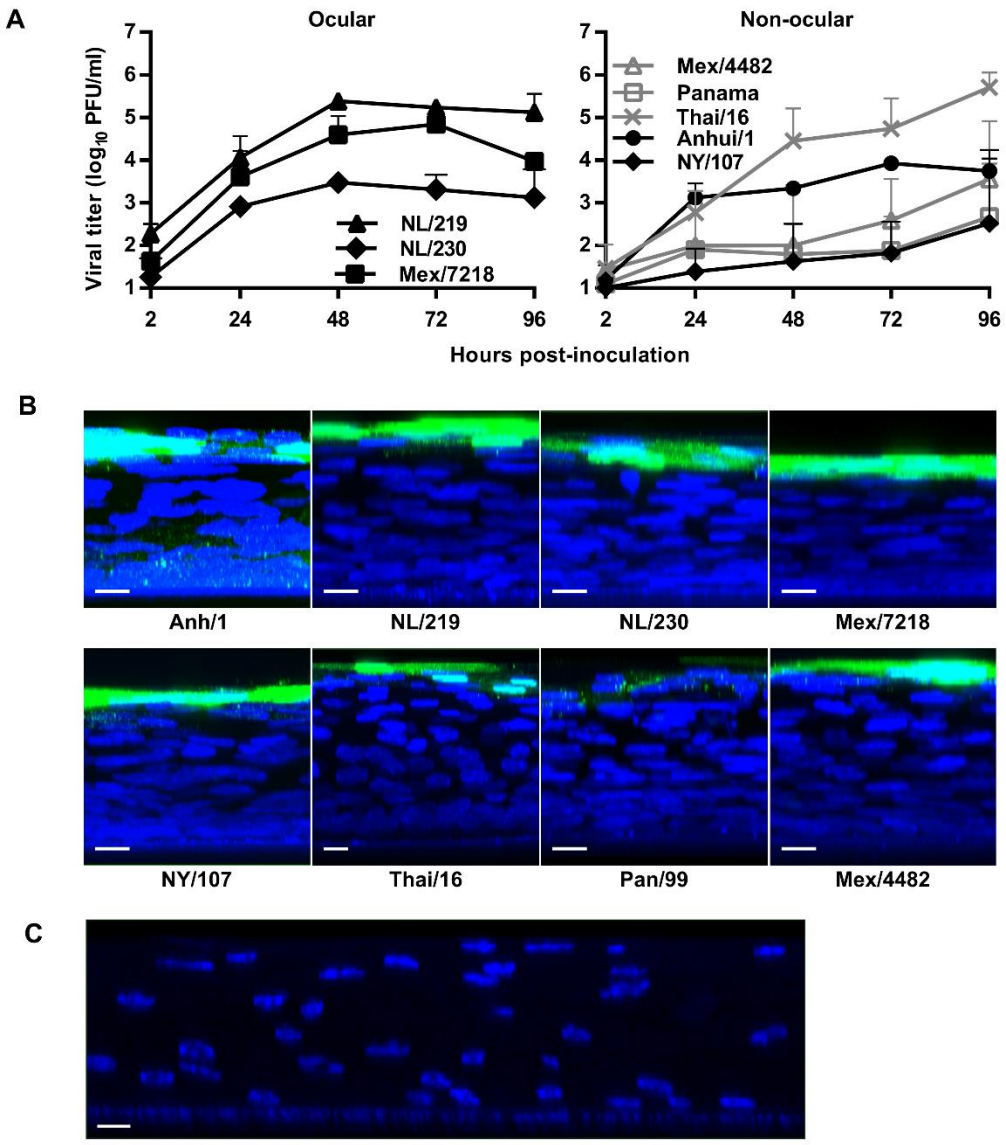

Figure 7. Replication and spread of influenza virus in corneal tissue constructs. Constructs were infected with the indicated viruses at an MOI of 0.01 using traditional liquid-based methods. A) Curves for NL/230, Anh/1, Thai/16, and Pan/99 viruses are repeated from Fig. 6 for convenience. Points for other viruses represent mean + SD of duplicate wells. B) Cells were fixed 96 hours post-infection and stained with anti-NP antibody (green) and DAPI (blue). A maximum intensity projection across the x-axis is

shown. C) Nuclear density across a single focal plane. Orthogonal view of a z-stack of an uninfected construct stained with DAPI. Scale bars=10μm.

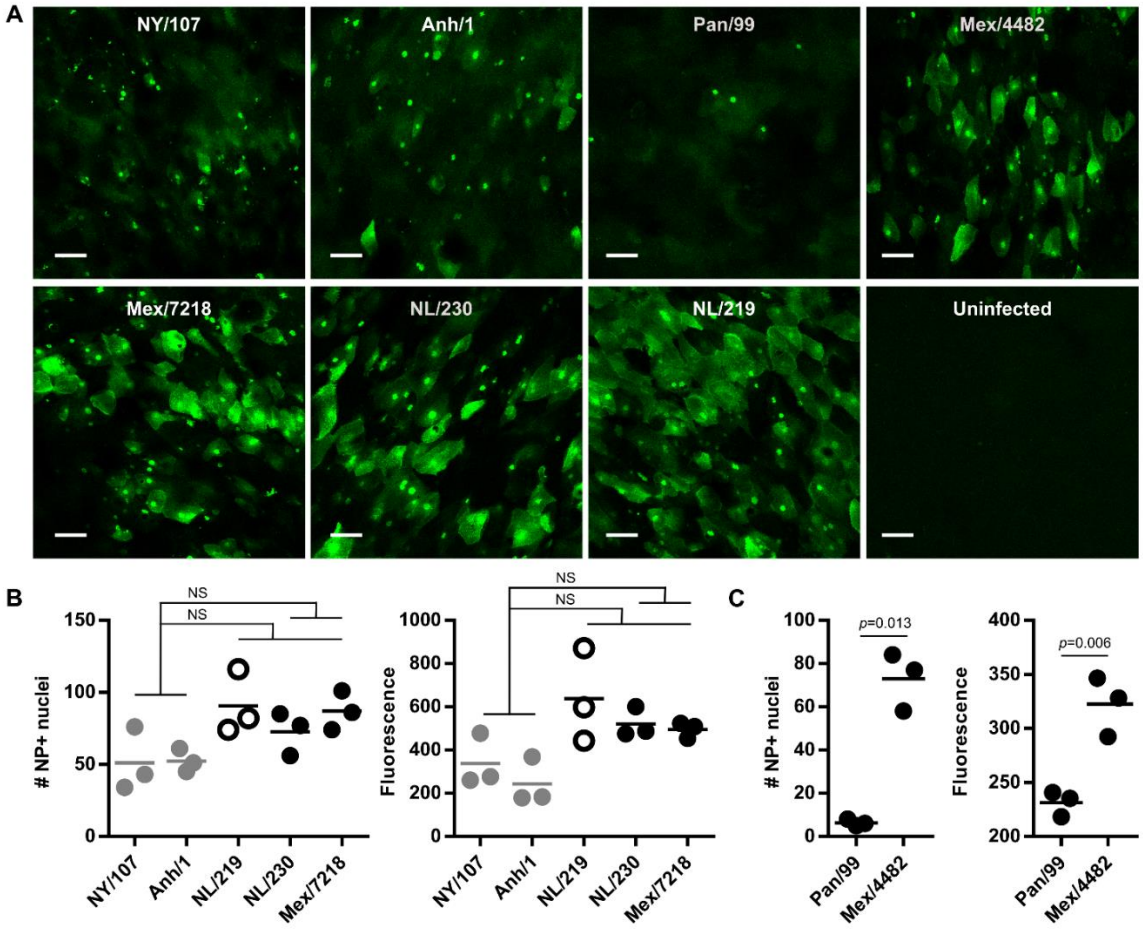

Figure 8. Single cycle infection of corneal tissue constructs. Corneal constructs were inoculated at an MOI of 2, then fixed 8 hours post-infection and stained with antibody against the viral nucleoprotein (green). A) Representative 10x fields. Scale bar = 100μm. B) Number of NP+ nuclei and fluorescence activity per field (n=3) in constructs infected with H7 viruses. Statistical significance determined by one-way ANOVA followed by pre-planned post-test performed using the multcomp package in R (67). C) Number of NP+ nuclei and fluorescence activity per field (n=3) in constructs infected with human influenza viruses. Statistical significance was determined by t-test. NS, not significant ($p > 0.05$).

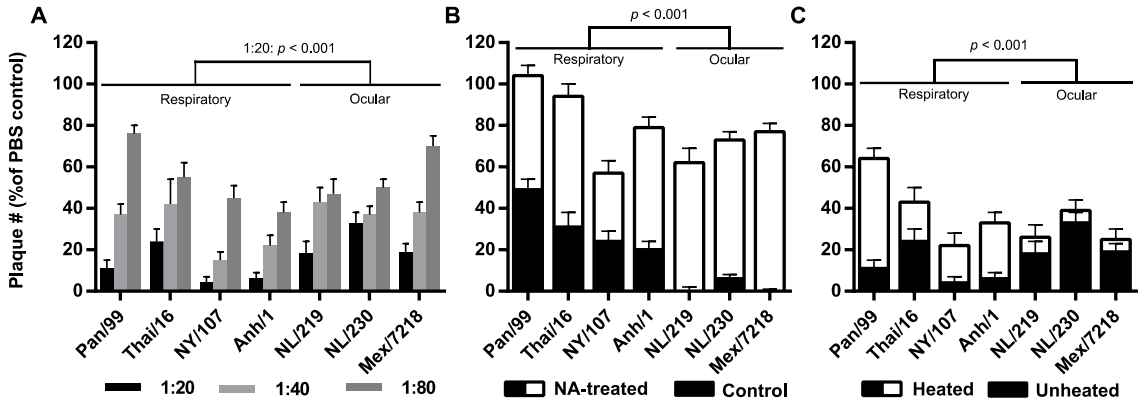

Figure 9. Inhibition of influenza virus by human tears. Virus was mixed with human tears or PBS and incubated at room temperature for one hour, then titrated via plaque assay. Binomial mean of data from three experiments + 95% confidence interval is shown. Statistical significance was determined via chi-square test. A) Human tears reduce viral viability in a dose-dependent manner. Legend indicates the total dilution of tears after mixing with virus. The indicated statistical comparison is for the lowest dilution (1:20). B) Effect of exogenous neuraminidase treatment on inhibitory effect of human tears (1:20). Total bar height indicates plaque number for tears which were pre-treated with exogenous neuraminidase before mixing with virus. Black region indicates combined effect of controls. Statistical comparison is for the difference between neuraminidase pre-treated tears and control (white region). C) Effect of heat-treatment on inhibitory effect of human tears (1:20). Tears were incubated at 37°C for one hour, followed by 30 minutes at 65°C. Total bar height represents the percentage of virus which remained infectious after incubation with heat-heated tears. Black region indicates unheated control. Statistical comparison is for the difference between the percentage of virus that remained infectious after incubation with heated and unheated tears (white region).

Supplemental Information

Table S1. Exact exposure doses for each influenza virus shown in Figure 3

Virus	Exposure dose (PFU/well)
Mex/7218	15
Italy/3	9.7
NL/230	7.3
NL/219	7.3
Anh/1	8.5
NY/107	8.1
Bang/5487	154
Thai/16	9.7
Pan/99	3.6
Mex/4482	6.5

Table S2. Antibodies used in this study

Target	**Supplier**	**Catalog #**
MUC1	Cell Signaling Technology, Inc	14161
MUC4	ThermoFisher Scientific	35-4900
MUC16	ThermoFisher Scientific	MA5-11579
ZO-1	ThermoFisher Scientific	339194
Influenza nucleoprotein	International Reagent Resource	FR-51

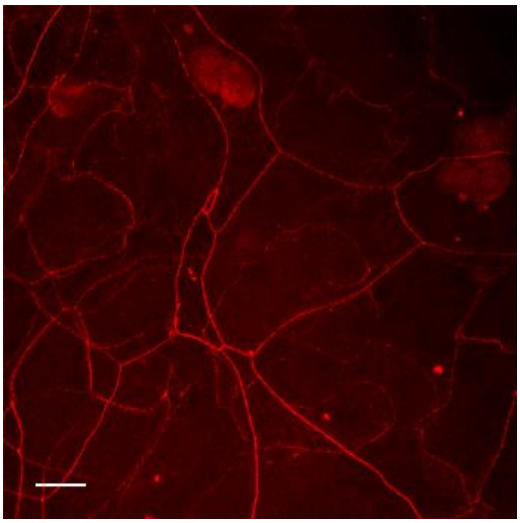

Figure S1. Tight junctions in corneal tissue constructs. Constructs were fixed and stained with antibody to the tight junction protein ZO-1 (red). Scale bar = 20 μm

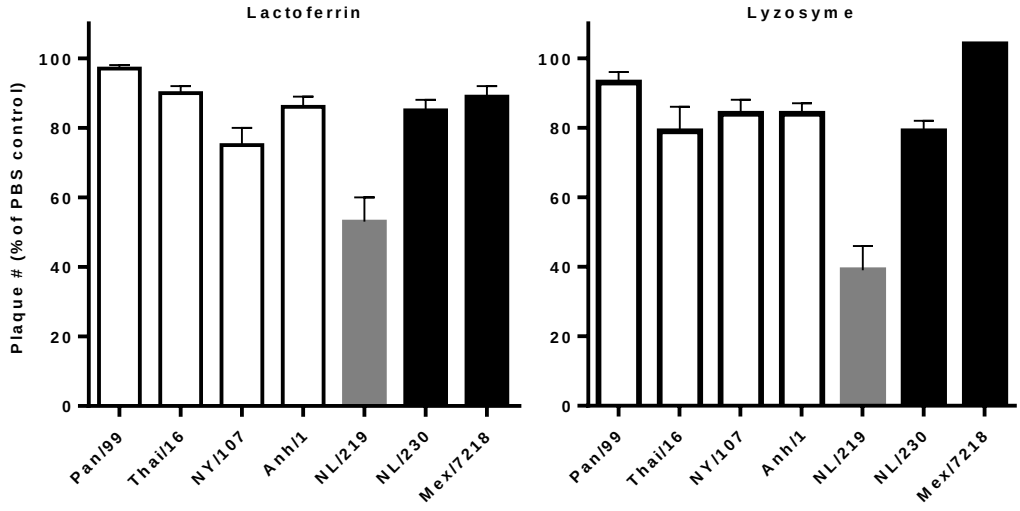

Figure S2. Influenza virus inhibition by human lactoferrin and lysozyme. Lyophilized human lactoferrin or lysozyme was resuspended in PBS at a concentration of 0.15 mg/mL, then mixed with an equal volume of virus. The mixture was incubated at room temperature for one hour, then titrated via plaque assay. Binomial mean of data from three experiments + 95% confidence interval is shown. White bars, respiratory viruses; black bars, ocular viruses.

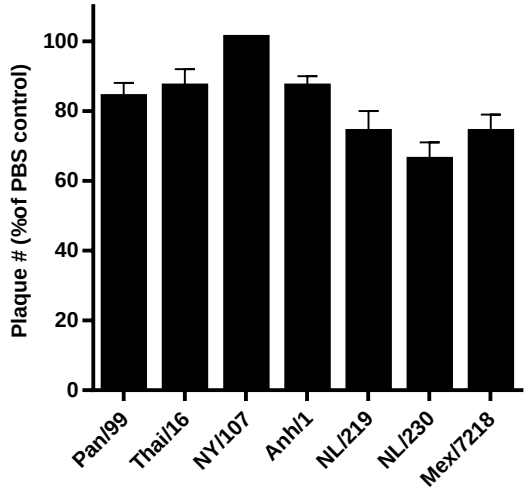

Figure S3. Heat-treated neuraminidase reduces influenza virus plaque assay titers. *V. cholera* neuraminidase at a concentration of 0.02 U/mL in PBS was incubated at 37°C for 60 minutes followed by 65°C for 30 minutes, then mixed with an equal volume of virus and incubated at room temperature for one hour before titration by plaque assay. Bars indicate binomial mean of data from three experiments + 95% confidence interval.

Chapter 4: Discussion

The increasing frequency of human infection with zoonotic influenza viruses provides cause for concern that one such virus might develop the ability to spread efficiently between humans. Analyses based on our current understanding of determinants of this capacity indicate that this may require only a handful of amino acid changes for this to occur (125-129). Given the risk posed by a potential pandemic, it is important that we understand the similarities and differences between these viruses and their seasonal counterparts. Tropism is one trait that differs significantly between viruses, and which plays a critical role in pathogenesis, but our knowledge of its determinants remains incomplete. We therefore sought to improve our understanding of tissue tropism in humans using novel methodology and non-canonical cell types.

In chapter 2, we show that cultured epithelial cell monolayers are susceptible to infection by low doses of aerosolized influenza virus. Infection of Calu-3 cells by aerosol inoculation was possible even when cells were cultured at air-liquid interface, resulting in the accumulation of a thick mucin layer atop the cells. In primary human alveolar epithelial cells, we observed differences in outcome between aerosol and liquid inoculation for Pan/99 and Anh/1, but not Thai/16 viruses. In our initial experiments, both Pan/99 and Anh/1 viruses replicated productively after liquid, but not aerosol inoculation, although similar multiplicities of infection were used in both cases. Upon repetition, Pan/99 virus could be detected at low levels in the supernatant following aerosol inoculation, but titers peaked at 24 hours post-infection before decreasing, indicating that if replication was responsible for the increase in viral concentrations in the supernatant between 2 and 24 hours post-infection, it was not sustained. Once again,

Anh/1 virus could not be detected in the supernatant at any timepoint following aerosol inoculation, though titers reached over 10^4 PFU/mL by 96 hours post-infection in wells exposed to a traditional liquid inoculum. Although the data are not included in the published manuscript, this experiment also compared the results of liquid and aerosol inoculations for several additional viruses. The seasonal H1N1 virus A/Brisbane/59/2007 failed to replicate in cells inoculated by either route, while NL/219 and two H5N1 viruses grew to high titers in all cases. NL/230 virus showed a pattern similar to that of Pan/99 virus, as did the variant H3N2 virus A/Indiana/8/2011 (Indiana/8). Additional repetition will be necessary to determine whether the phenotype of NL/230, Pan/99, and Indiana/8 viruses is consistent across experiments. We are more confident in the lack of Anh/1 following aerosol inoculation as we have reproduced this finding on several occasions, and because the failure to detect any infectious virus in the cell supernatants does not raise the question of whether or not replication is responsible for the transient low titers detected with the other viruses.

This unexpected result opens up several additional lines of possible inquiry. We initially suspected that Anh/1 virus was failing to replicate productively after aerosol inoculation because it was not able to infect cells. Because the cell culture conditions were identical across inoculation methods, we assumed that events downstream of infection would be not affected by the route of viral delivery, but it seemed possible that the aerosol delivery was somehow interfering with the ability of virions to bind to the surface of susceptible cells or with the endocytosis process. To confirm this hypothesis, we performed qRT-PCR with primers for the viral M gene on RNA extracted from the cell monolayers 24 hours after inoculation. Our results, however, provided evidence to

the contrary: at least 10^4 copies were present in each well, though no M gene copies were detected in our PBS-inoculated controls. Because mRNA is the first of the three viral RNA species produced, we can infer its presence within infected cells, but cannot determine whether cRNA and vRNA were generated.

We next investigated viral infectivity using immunofluorescent staining approaches. A pilot experiment demonstrated that viral nucleoprotein (NP) could be detected in Anh/1-exposed cells fixed 24 hours post-inoculation, though no virus was detectable in the supernatant of either the fixed cells or of identical wells which were incubated for an additional three days. However, NP staining was much more abundant in a well inoculated with Thai/16 virus. We propose that this is because cells infected by aerosolized Anh/1 virus do not produce infectious virus, meaning that only cells which were infected at the start of the experiment stain NP+. In contrast, many infectious Thai/16 virions are released from each infected cell and can initiate infection in additional naïve cells, resulting in an increased number of NP+ cells after several replication cycles. Repeating this experiment with a greater number of wells, some of which are fixed after only one replication cycle (approximately 6 hours post-infection) and others of which are fixed after multiple cycles (eg. at 24 hours post-infection) would show whether this is indeed the case; we would expect the number of Anh/1 positive cells to remain the same, or to decrease due to death of infected cells between 6 and 24 hours whereas the number of NP+ cells in wells exposed to Thai/16 and fixed at 24 hours post-inoculation would be much higher than in wells fixed only 6 hours after inoculation.

On the basis of present data, we can conclude that aerosol inoculation results in an interruption of the viral replication cycle of Anh/1 virus somewhere between the

translation of viral proteins and the release of infectious virus. To further narrow down the processes affected by inoculation method, follow-up studies should assay the supernatants of cells inoculated with Anh/1 for viral RNA in order to determine whether infected cells are releasing non-infectious virions or no virions at all. Strand-specific PCR targeting the viral M gene could also be performed on cellular RNA to see whether or not negative-sense RNA (ie. viral RNA) is produced.

Regardless of exactly which step of viral replication is disrupted, the question of the mechanism by which inoculation route affects replication events late in the replication cycle remains. One possibility is that the airflow associated with aerosol inoculations is disruptive to the cells in some way and therefore activates innate immune responses, which induce an anti-viral state, interfering with replication. The fact that the disruption appears to occur relatively late in the replication cycle is consistent with the need for time to induce expression of antiviral proteins. A related explanation is that because the aerosolization process inactivates some virions, and inoculation doses are based on number of infectious virions, cells inoculated by the aerosol route are exposed to a higher number of non-infectious virions than are liquid inoculated cells. Since non-infectious virions are able to induce innate immunity, the greater total number of virions to which aerosol-inoculated cells are exposed might trigger a more vigorous antiviral response, or induce it more quickly (130).

A related, and more difficult question is why the differences between aerosol and liquid inoculation are evident in alveolar, but not other cell types (*eg.* Calu-3, primary human corneal) and only in the case of select viruses. While the level of viral inhibition by innate immune responses could potentially differ between viruses as a result of

polymorphisms in the NS1 or PA-X proteins, other potential mechanisms (*eg*. endocytosis by different pathways) would presumably affect all viruses equally. Further exploration of which viruses replicate in alveolar cells after liquid, but not aerosol inoculation, and whether this also occurs in other cell types may point toward the pathway by which aerosol inoculation prevents replication in otherwise permissive cells.

In chapter 3, we investigated whether the same phenomenon occurred in human corneal epithelial cells. This was of interest as only a subset of the influenza viruses which replicate well *in vitro* tend to cause ocular infections *in vivo*, a discrepancy we suspected might be an artifact of the use of artificial inoculation methods. However, the influence of inoculation method on infection and replication was minimal. Similarly, neither culture at 33°C nor the presence of mucins on the apical surface of differentiated tissue constructs favored ocular isolates. Together, these findings suggested that something prevents virus that travels to the ocular surface from infecting the cells there.

Burnet and Beveridge reported in a 1945 paper that human tear fluid inhibited hemagglutination by two laboratory influenza strains and a contemporary human virus that had been recently isolated (131). They also found that incubating virus with tear fluid led to a "limited reduction in viral titers" as determined by chorioallantoic titration when the incubation was carried out at 37°C, but had no effect if the incubation occurred in a refrigerator. However, no one has followed up on these findings since they were published. We hypothesized that inhibition by the tear film might explain why viruses which replicate well in ocular cells under a variety of conditions rarely cause conjunctivitis in humans. Using a panel of more recently isolated human and avian viruses, we confirmed that human tears both inhibited viral hemagglutination and reduced

infectivity, as measured by plaque assay in MDCK cells. More importantly, we demonstrated that the post-incubation reduction in plaque number was smaller for conjunctivitis-associated isolates than for other virus strains, suggesting that the capacity maintain infectivity while in contact with the tear film may be an important determinant of ocular tropism *in vivo*.

Removal of sialic acid from tears by an exogenous neuraminidase significantly reduced their inhibitory effect for all viruses. This is not surprising given that mucins are known to be present in tears and that mucus is known to inhibit influenza infection by binding virus (132). However, the presence of sialic acid can only partially account for the inhibitory effect of human tears, and it does not explain why conjunctivitis-associated isolates are less inhibited by tears than isolates not associated with conjunctivitis given that the former were more affected by sialic acid. We provide evidence for the importance of a heat-labile factor or factors to the differential inhibition, but were not able to identify it. It seems possible that the complement system plays a role: complement proteins are known to be present in tears, are heat-labile, and can neutralize free virions by lysis or by mediating viral aggregation. Complement deposition on influenza virions by way of the alternative pathway has been shown, and the lectin pathway could presumably lead to activation as mannose-binding lectin is able to bind to the viral HA (133). However, there is conflicting evidence about whether either of these pathways leads to virus neutralization by serum (134, 135).

Interestingly, Gilbertson et al. recently deduced the presence in mouse saliva of a non-sialic acid inhibitor of the viral neuraminidase to which susceptibility differed greatly between strains (136). By interfering with the neuraminidase of susceptible

viruses, the inhibitor curtailed the viral release process, thereby preventing the viral spread to adjacent cells required for plaque formation. Although the heat stability of the Gilbertson inhibitor is not reported, it does not represent a likely candidate for the inhibitory tear component we observed as it acts late in the replication cycle, when it would not have been present in our plaque reductions assays as we wash the cells after inoculation and prior to adding the agar overlay.

In summary, we have shown that cultured epithelial cell monolayers can be infected by exposure to droplet aerosols containing low concentrations of influenza virus. Not only does this facilitate novel *in vitro* tropism studies, but it also has the potential to enhance *in vivo* studies of aerosolized influenza virus. While there is great interest in the amount and nature of virus exhaled by infected ferrets, collection and detection of live virus has proved difficult. Detection and analysis of exhaled virions using a cell culture readout, as we characterize in chapter 2, may offer a more sensitive method of detecting live virus released from infected animals than currently employed approaches (eg. use of an impinger or impactor for collection of virus in liquid or gelatin). Though use of an aerosolized inoculum did not substantially alter viral infection or replication in human corneal epithelial cells, restriction of certain viruses following aerosol, but not liquid inoculation, demonstrates the importance of using aerobiological approaches in the laboratory. Similarly, viral infection and replication did not substantially differ between primary corneal cells grown in a monolayer and differentiated human corneal tissue constructs, but the influence of tear fluid on viral infectivity underscores the need for increased complexity of *in vitro* studies. Influenza virus tropism is clearly a multifactorial

trait: as highlighted throughout this dissertation, the laboratory approach to studying this property should be similarly diverse.

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