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In vitro exposure system for study of aerosolized influenza virus

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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 human 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.

1. 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, 2011, 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*

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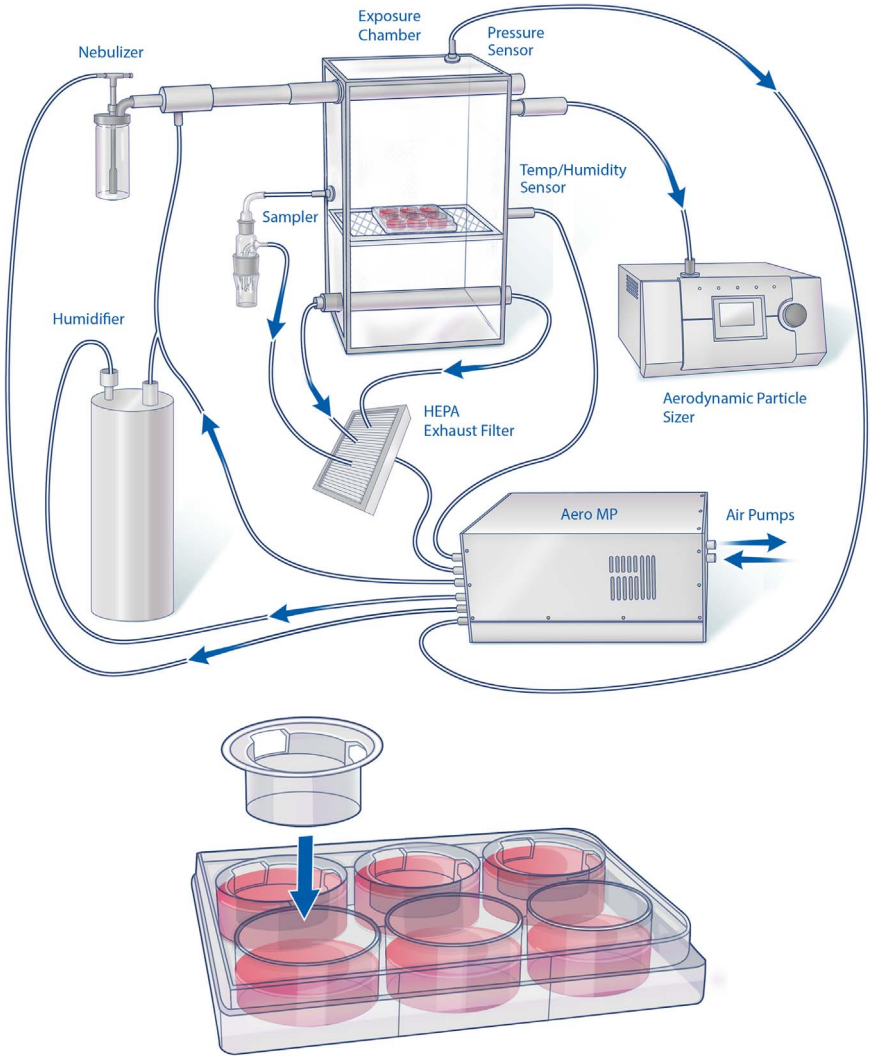

Fig. 1. Graphic representation of aerosol system for in vitro use. Depiction of human cells cultured on transwell inserts and exposed to aerosolized influenza virus using a previously characterized system (Gustin et al., 2011). Cell culture dishes rest in the exposure chamber on a wire shelf under air-liquid interface conditions for the duration of the exposure. Inset, individual transwell inserts are transferred to sterile plates once removed from the exposure chamber.

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 avian influenza (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.

2. Materials and methods

2.1. 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).

2.2. 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 0.4 μm pore size (Corning) and grown to confluence under submerged conditions. After