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Live attenuated influenza vaccine strains elicit a greater innate immune response than antigenically-matched seasonal influenza viruses during infection of human nasal epithelial cell cultures

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ABSTRACT

Influenza viruses are global pathogens that infect approximately 10–20% of the world's population each year. Vaccines, including the live attenuated influenza vaccine (LAIV), are the best defense against influenza infections. The LAIV is a novel vaccine that actively replicates in the human nasal epithelium and elicits both mucosal and systemic protective immune responses. The differences in replication and innate immune responses following infection of human nasal epithelium with influenza seasonal wild type (WT) and LAIV viruses remain unknown. Using a model of primary differentiated human nasal epithelial cell (hNECs) cultures, we compared influenza WT and antigenically-matched cold adapted (CA) LAIV virus replication and the subsequent innate immune response including host cellular pattern recognition protein expression, host innate immune gene expression, secreted pro-inflammatory cytokine production, and intracellular viral RNA levels. Growth curves comparing virus replication between WT and LAIV strains revealed significantly less infectious virus production during LAIV compared with WT infection. Despite this disparity in infectious virus production the LAIV strains elicited a more robust innate immune response with increased expression of RIG-I, TLR-3, IFNβ, STAT-1, IRF-7, MxA, and IP-10. There were no differences in cytotoxicity between hNEC cultures infected with WT and LAIV strains as measured by basolateral levels of LDH. Elevated levels of intracellular viral RNA during LAIV as compared with WT virus infection of hNEC cultures at 33 °C may explain the augmented innate immune response via the up-regulation of pattern recognition receptors and down-stream type I IFN expression. Taken together our results suggest that the decreased replication of LAIV strains in human nasal epithelial cells is associated with a robust innate immune response that differs from infection with seasonal influenza viruses, limits LAIV shedding and plays a role in the silent clinical phenotype seen in human LAIV inoculation.

1. Introduction

Influenza viruses are global pathogens that infect approximately 10–20% of the world's population each year contributing to an excess in morbidity and mortality [1]. Annual immunization, including the Live Attenuated Influenza Vaccine (LAIV) and the trivalent inactivated influenza vaccine (TIV), remain the world's best defense against seasonal influenza.

LAIV strains are nasal vaccines that actively replicate in the human nasal epithelium and elicit a protective mucosal immune response without causing clinical disease. The initial LAIV was originally developed by cold passage of a strain of influenza (H2N2; A/Ann Arbor/6/60) in a primary chicken kidney cell line. The virus isolated from these conditions exhibited three novel phenotypes including the ability to replicate at cooler temperatures (cold adapted; CA), restricted replication at temperatures above 39 °C (temperature sensitive; TS), and lack of clinical disease in animal models and human experiments (attenuation; att) [2,3]. Previous studies, comparing cold adapted LAIV strains and antigenically matched influenza viruses, in non-human and immortalized cell lines have identified mutations in the PB1, PB2, M, PA, and NP

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segments of the LAIV which have been associated with each of these phenotypes. While specific mutations have been associated with decreased replication or attenuation in animal models the actual mechanisms, including how the live attenuated influenza vaccine is able to elicit a protective immune response despite lower replication at the true site of infection – the human nasal epithelium remain unknown.

Human nasal epithelial cells are of particular importance in influenza virus and vaccine infection as they are not only the principle cells infected but are also key cells responsible for initiating an effective yet controlled immune response [4,5]. Influenza virus infection of nasal epithelial cells induces an innate antiviral response in addition to the recruitment of adaptive immune cells. An initial epithelial cell-specific innate immune response is elicited through the interaction of host cell pattern recognition receptors (PRRs; including TLR3, TLR7, and RIG-I) and viral RNA. The recognition of viral RNA by PRRs result in the production of Type I IFN that lead to induction of down stream signaling molecules such as STAT-I as well as antiviral interferon stimulated genes including myxovirus resistance gene A (MxA) [5]. Together these innate immune mediators act to limit virus replication. Additionally, engagement of PRRs by influenza viral RNA also trigger the production and release of the neutrophil chemokine IL-8 and IP-10 which recruit Th1 activated T cells, NK cells, and dendritic cells into the airway [6]. It is clear that the innate immune response directed by the nasal epithelium following influenza virus or vaccine infection plays a key role in limiting early virus replication and orchestrating an effective adaptive immune response.

In this study we characterize the differences in virus replication and the innate immune response to infection with a WT seasonal influenza virus and an antigenically-matched cold adapted (CA) LAIV strain in the principal cells infected by influenza viruses, human nasal epithelial cells, in order to begin to understand how LAIV viruses are able to replicate in the human nasal epithelium yet not cause clinical disease.

2. Material and methods

2.1. Human nasal epithelial cell procurement and culture

Primary differentiated human nasal epithelial cells (hNECs) were obtained and cultured as previously described [7–11]. Briefly, nasal epithelial cells were obtained by gently stroking the inferior superficial surface of the inferior turbinate several times with a Rhino-Probe curette (Arlington Scientific, Arlington TX), which was inserted through a nasoscope. hNECs were obtained from healthy, non-smoking, volunteers ($n = 7$) aged 18–55 years who identified themselves as healthy without a diagnosis of asthma or smoking related disorder. This protocol was approved by the Institutional Review Board for Biomedical Research of the University of North Carolina at Chapel Hill School of Medicine (IRB No 09-0716).

Primary hNECs were expanded to passage 2 in bronchial epithelial growth medium (Lonza, Walkersville, MD), plated on collagen-coated filter supports with a 0.4 μm pore size (Corning, Tewksbury, MA). Upon confluence, air-liquid-interface (ALI) conditions were created to promote differentiation. Muco-ciliary differentiation was achieved 21–28 days after ALI.

2.2. Viruses

Wild-type viruses were propagated on MDCK cells in Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO) containing 4 μg/ml *N*-acetyltrypsin (Sigma), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 0.3% bovine serum albumin (BSA; Sigma). The viruses used in this study included a seasonal Wild Type (WT) virus, A/California/10/78 (H1N1), and an antigenically matched cold-adapted (CA) LAIV strain, A/California/10/78 CR37 CL1, which were kindly provided by K. Subbarao at the NIAID/NIH.

2.3. Virus infections

High-multiplicity of infection (MOI) growth curves were generated by infecting hNEC cultures in 12-well plates at an estimated MOI of 10 $TCID_{50}$/ml per cell. Inoculums were calculated based on known concentration of stock viruses to achieve an estimated MOI of 10 and then confirmed by back-titer in parallel with apical samples at designated time points. Inoculums and stock virus were also tittered by hemagglutination assay (data not shown). Viruses were diluted in DMEM (Sigma) containing penicillin, streptomycin, and 0.3% BSA on the apical surface for 1 h at room temperature. The inoculum was then aspirated and the apical and basolateral chambers were washed twice with PBS. Basolateral media composed of equal proportions BEGM (Lonza) and DMEM (Gibco, Grand Island, NY) was added and the cultures were incubated at 33 °C. Viral infections of hNEC cultures were performed at 33 °C to faithfully represent the temperature of the human nasal epithelium [12,13]. At the indicated times, basolateral supernatants (for cytokine detection) were collected, apical chambers were washed with 200 μl of DMEM containing penicillin, streptomycin, and 0.3% BSA (apical supernatant), and both were stored at −80 °C until analysis.

2.4. Virus titers

Infectious virus titers, at the indicated time points, were measured using a $TCID_{50}$/ml assay as previously described using Madin Darby Canine Kidney Cells (MDCK) at 33 °C [14].

2.5. Cytokine quantification

Bi-directional hNEC secretion of cytokines was measured by sampling apical and basolateral chambers separately. Apical and basolateral supernatants were analyzed for IL-8 and IP-10 using commercially available ELISA kits (BD OptEIA, Biosciences, San Diego, CA). Lower limits of detection were as follows: IL-8 = 3.1 pg/ml and IP-10 = 7.8 pg/ml. Cytokines were assessed at 24 h post infection in order to ensure that the innate immune response being measured was that to virus replication rather than inoculum.

2.6. Real-time quantitative RT-PCR

Total RNA was isolated from hNEC cultures using TRizol (Invitrogen, Grand Island, NY) according to manufacturer's instructions. cDNA synthesis and real-time quantitative PCR (qPCR) for RIG-I, TLR-3, IFN-b, IRF-7, MxA, and Stat-1 were performed as previously described [15–17]. Differences in gene expression results were determined with the $\Delta\Delta Ct$ method and normalized first to β-actin then to mock-infected cultures and presented as fold change in gene expression. Cell associated influenza viral mRNA was determined by qRT-PCR using primers specific for the influenza A virus M segment in paired samples. Differences in influenza A virus M segment detected were determined with the $\Delta\Delta Ct$ method and normalized to a WT sample, since no RNA was detected in mock-infected samples.

2.7. Statistical analysis

Experiments conducted in hNEC cultures from seven healthy donors were paired for analysis. Replication kinetics for wild-type seasonal viruses and antigenically-matched cold-adapted vaccine strains were compared using two-way analysis of variance