Human coronaviruses 229E and OC43 replicate and induce distinct antiviral responses in differentiated primary human bronchial epithelial cells

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INTRODUCTION

In addition to recently emerged zoonotic coronaviruses severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1; 2002), Middle East respiratory syndrome (MERS)-CoV (2012), and SARS-CoV-2 (2019), there are four known endemic human CoVs: NL63, HKU1, OC43-CoV (OC43), and 229E-CoV (229E). 229E and OC43 were identified during the 1960s (19) and since then, they have been implicated in causing mild respiratory disease and, after rhinoviruses, are a leading cause of common colds (10–30%) (14, 23, 26).

NL63-CoV (NL63) (27) and HKU1-CoV (HKU1) (32) were later discovered when the SARS-CoV pandemic generated intensified screening for CoVs. CoVs are further divided into four genera based on phylogeny: alpha, beta, gamma, and delta. 229E and NL63 belong to the alpha genera while OC43, HKU1, SARS-CoV-1, SARS-CoV-2, and MERS-CoV are betacoronaviruses (5, 6, 17, 28).

Little is known about replication and innate immune responses to human CoVs in airway epithelial cells. In vitro studies utilizing bronchial epithelial cell (BEC) cultures differentiated at the air-liquid interface (ALI) have reported that 229E host receptor aminopeptidase N is found predominately on nonciliated cells (29). Although the functional cellular receptor for OC43 remains unknown, the OC43 S protein has been shown to use the sugar 5-N-acetyl-9-O-acetylaminoacetic acid (Neu5, 9Ac2) as a receptor (15, 21, 22). OC43 has been shown to infect ciliated cells via Neu5, 9Ac2 expression on this cell type (29).

BECs express germ-line encoded pathogen-recognition receptors (PRRs) (24), which upon recognition of a pathogen-derived molecule (viral RNA), leads to the expression of interferons (IFNs), interferon gamma-induced protein (CXCL10) (30), and proinflammatory cytokines such as interleukin-6 (IL-6) (25). Type I interferons (IFN-α/β) and type III IFNs (IFN-λ1-3) are particularly important in the innate antiviral response with type III IFNs specifically involved in epithelial innate immune protection to respiratory viruses (9) that involves expression of interferon-stimulated genes (ISGs) such as viperin, 2′,5′-oligoadenylate synthetase (OAS), and protein kinase R (PKR) (7).

In the current study, we developed two human CoV infection models utilizing human primary BECs (pBECs) differentiated at the ALI to investigate the replication kinetics and epithelial innate immune response to OC43- and 229E-CoVs. This study showed that these CoVs have distinct replication kinetics and induce different innate antiviral cytokine profiles. This study supports the use of this in vitro model to investigate interventions targeting viral replication or host antiviral immunity as well as providing insights into mechanisms of coronavirus-induced disease.

MATERIALS AND METHODS

Ethics statements, donor recruitment, and pBEC collection. pBECs were provided by P. A. B. Wark (The University of Newcastle), obtained from healthy nonsmoking donors during bronchoscopy, with

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an Epithelial Volt/Ohm (TEER) Meter (World Precision Instruments). Trans-epithelial electrical resistance (TEER) was measured weekly using this instrument and assessment of trans-epithelial electrical resistance. The presence of ciliated epithelium, mucus-producing cells, pseudostriatified columnar basal compartment (beneath the transwell insert) without apical media. The quality of cultures was confirmed by differentiation in the basal compartment. 

Once confluent, rhEGF was added to the cultures until confluent (at least 3 days in both apical and basal compartments). Once confluent, rhEGF concentration was changed to 0.5 ng/mL during the ALI phase for differentiation in the basal compartment (beneath the transwell insert) without apical media. The quality of cultures was confirmed by the presence of ciliated epithelium, mucus-producing cells, pseudostriatified structure, and assessment of trans-epithelial electrical resistance. Trans-epithelial electrical resistance (TEER) was measured using an Epithelial Volt/Ohm (TEER) Meter (World Precision Instruments).

Coronavirus propagation. For OC43 (ATCC number VR-1558) and 229E (ATCC number VR-740), OC43 passage history is unknown, while passage history of 229E shows two passages. American Type Culture Collection (ATCC) indicates that OC43 was propagated on HCT-8 cells and 229E on MRC-5 cells. The ATCC-supplied OC43 titer was 2.8 \( \times 10^8 \) TCID50/mL and 229E titer was 2.8 \( \times 10^7 \) TCID50/mL. Upon receiving the ATCC stocks both viruses were passaged three times in MRC-5 cells to generate working stocks: OC43 titer was 2 \( \times 10^8 \) TCID50/mL and 229E titer was 2 \( \times 10^7 \) TCID50/mL and used for studies according to WHO guidelines. MRC-5 cells were utilized for TCID50 assays using the Karber method.

Coronavirus infection. Infection time-courses for each donor were conducted independently with one well per treatment using the same stocks of OC43 and 229E for all experiments. pBECs were starved basally with BEBM Minimal media ((Lonza; BEBM + 1% ITS, 0.5% newborn bovine serum albumin (final concentration 0.5 mg/mL), amphotericin B (final concentration 250 \( \mu \)g/mL), all-trans retinoic acid (30 ng/mL), hydrocortisone, 0.1% bovine insulin, 0.1% epinephrine, 0.1% transferrin, 0.4% linoleic acid, 2% penicillin-streptomycin and 1% fungizone) for 24 h before infection. ALI cultures were apically infected with OC43 [multiplicity of infection (MOI) 0.1], 229E (MOI 0.1) or mock-infected with starvation media controls for two hours at 33°C (OC43) or 35°C (229E). Transwells were washed with PBS to remove unbound virus and starvation media was apically applied on all wells. pBECs were harvested at 0, 24, 96, or 168 h postinfection. During the time course, basal media was replenished at 48 and 96 h.

Sample collection and analysis from ALI cultures. Apical media was removed and stored at −80°C for viral infectivity and downstream protein analyses. Cytometric bead array (BD Bioscience) was performed to quantify CXCL10 and IL-6 on a FACSCanto II cytometer (BD BioSciences). Data were analyzed using FCAP Array software. IFN-β (PBL Assay Science) and IL-29/IL-28B (IFN-κ1/3) (R&D Systems) were measured by ELISA using the FLUostar OPTIMA Multi-Detection Plate Reader (BMG Labtech).

Table 1. Primers

| Target Gene | Forward Primer | Reverse Primer | Probe |
|-------------|----------------|----------------|-------|
| N-229E      | 5′-CAGTCAAATGGGGCTATGCA | 5′-AAAGGGGCTAAAAAGAAGTATTTCTT | 5′-FAM-CCCTGACGCACGGTGTTGCTCA-TAMRA |
| N-OC43      | 5′-CGATGGGGCTATTCCGACGTTA | 5′-CTCTCTGGACATATATATATATG | 5′-FAM-TGGGGCCACGGCGGAGTTTCTC-TAMRA |
| IHV         | 5′-AGCATGGGCTATTCCGACGTTA | 5′-CTCTCTGGACATATATATATATG | 5′-FAM-TGGGGCCACGGCGGAGTTTCTC-TAMRA |
| Viperin     | CAACAAAGAAGGTTGCTGAGTGCTT | AAGCCATATATATATATATATG | 5′-FAM-TGGGGCCACGGCGGAGTTTCTC-TAMRA |
| OAS         | CCGTGAGGCTATTCCGACTTGGCTT | CCGTGAGGCTATTCCGACTTGGCTT | 5′-FAM-TGGGGCCACGGCGGAGTTTCTC-TAMRA |
| PKR         | AAAGGGGCTATTCCGACTTGGCTT | CCGTGAGGCTATTCCGACTTGGCTT | 5′-FAM-TGGGGCCACGGCGGAGTTTCTC-TAMRA |

Table 2. Healthy donor characteristics

| Donor | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Median | IQR |
|-------|---|---|---|---|---|---|---|---|--------|-----|
| Age, yr | 62 | 78 | 62 | 48 | 74 | 47 | 76 | 53 | 62 | 23 | 25 |
| Sex   | F | F | M | M | M | F | M | F | F | |

IQR, interquartile range; F, female; M, male.

Fig. 1. Distinct replication kinetics of OC43 and 229E in differentiated bronchial epithelial cells from healthy donors. Infectivity of released virus collected at apical surface (A) and viral RNA expression (B) at 0, 24, 96, and 168 h postinfection. n = 8 healthy donors, median ± interquartile range (A and B). Log transformed data analyzed by 2-way ANOVA with Sidak’s multiple comparisons test (A and B). *P < 0.05, ***P < 0.001 increased in 229E vs. OC43, #P < 0.05 increased OC43 vs. 229E.
Transwells were collected in RLT buffer (Qiagen) containing 1% 2-mercaptoethanol (2ME) for molecular analyses and in RIPA buffer containing protease inhibitor cocktail (Roche) for protein analyses. RNA extraction was conducted according to manufacturer instructions (Qiagen, RNEasy Mini Kit). Purified RNA was quantitated by spectrophotometry (Nanodrop) and cDNA synthesized using the High Capacity cDNA Reverse Transcription Kit (ABI). For OC43 and 229E nucleocapsid gene PCR detection, standards were produced for quantitative PCR using plasmids. All qPCR analysis was conducted using the ABI 7500 Real Time PCR System. Targets included 18S, IFN-β, Interferon-stimulated gene-15 (ISG-15), and OAS. 

Capacity cDNA Reverse Transcription Kit (ABI). For OC43 and 229E RNA extraction was conducted according to manufacturer instructions containing protease inhibitor cocktail (Roche) for protein analyses. Purified RNA was quantitated by spectrophotometry (Nanodrop) and cDNA synthesized using the High Capacity cDNA Reverse Transcription Kit (ABI). All qPCR analysis was conducted using the ABI 7500 Real Time PCR System. Targets included 18S, IFN-β, Interferon-stimulated gene-15 (ISG-15), and OAS. Protein data were compared by two-way ANOVA with Sidak’s multiple comparisons test. Single time point data were analyzed using nonparametric Friedman test. A value of P < 0.05 was considered statistically significant.

RESULTS

Donor characteristics. Differentiated pBECs from healthy donors (n = 8) were grown at the air-liquid interface (ALI) and infected with OC43-CoV or 229E-CoV at a MOI of 0.1. Cells and supernatants were collected at 0, 24, 96, and 168 h after infection. One patient had 0 and 24 h time points only. Patient details are listed in Table 2.

Viral replication kinetics of OC43-CoV and 229E-CoV. Apical supernatants were measured for 229E and OC43 viral infectivity using the TCID50 assay in MRC-5 cells. Figure 1A shows the viral infectivity for OC43 and 229E over a 7 day (168 h) time course. 229E viral infectivity peaked at 24 h postinfection and was significantly (1,000 fold) greater than OC43 viral load at that time point. 229E virus production decreased after 24 h postinfection and was undetectable by 168 h postinfection. OC43 viral titers increased more slowly than 229E, peaking at 96 h postinfection with sustained production of infectious virus at 168 h postinfection that was significantly higher than 229E at this time. Figure 1B shows the viral RNA expression over the time course. Early (24 h postinfection) expression reflected the viral infectivity results, with 229E RNA synthesis peaking at 24 h, significantly (~1,000 fold) higher than OC43. OC43 RNA peaked at 96 h with levels remaining significantly lower than 229E. At 168 h postinfection 229E RNA production had reduced and was not significantly different from OC43 by this time.

Innate immune activation during 229E and OC43 infection. 229E infection induced significantly increased IFN-β protein expression at 96 h (~10-fold mock-infected cells) with levels decreasing by 168 h postinfection but remaining significantly elevated above mock-infected cells. In contrast, OC43 failed to induce IFN-β expression at any time (Fig. 2A). 229E infection also induced type III IFN-λ protein expression (~1,000-fold over mock-infected cells by 96 h and 168 h postinfection (Fig. 2B). No induction of IFN-λ was detected in OC43-infected BECs. Coinciding with peak expression of IFN-β, expression of ISGs viperin, OAS and PKR were also significantly increased at 96 h postinfection by 229E compared with mock-infected cells. OC43 infection did not induce ISG expression (Fig. 2C).

Fig. 2. OC43 and 229E coronaviruses induce different type I and III IFN response profiles in primary bronchial epithelial cells. IFN-β protein expression in apical supernatants (A), IFN-λ-1/3 protein expression in apical supernatants during 229E- and OC43-coronavirus (CoV) infection at 0, 24, 96, and 168 h postinfection (B), and viperin, 2′,5′-oligoadenylate synthetase (OAS), and protein kinase R (PKR) mRNA expression in whole cell lysates during 229E- and OC43-CoV infection at 0, 24, 96, and 168 h postinfection (C). n = 8 healthy donors, median with interquartile range (A–C). Data analyzed by 2-way ANOVA with Dunnett’s multiple comparison test (A–C). *P < 0.05, ***P < 0.001, ****P < 0.0001 229E compared with mock infected cells.
Consistent with type I/III IFNs, 229E infection induced CXCL10 protein expression that was significantly increased (100-fold) over mock-infected cells by 96 to 168 h post infection (Fig. 3A). CXCL10 was also detected in OC43 infection, but this was ~10-fold less than the magnitude of 229E induction and was not significantly higher than mock-infected cells. Apical supernatants were analyzed for the proinflammatory cytokine interleukin-6 (IL-6) (Fig. 3B). The induction of IL-6 protein during both the OC43 and 229E infection time course was minimal and comparable with mock-infected controls, however assessment at 96 h postinfection indicated a trend \((P = 0.2)\) for induction by 229E.

**DISCUSSION**

**229E and OC43 display distinct replication kinetics.** 229E and OC43-CoV infection in differentiated pBECs displayed distinct replication kinetics. OC43 replication steadily increased, with viral infectivity peaking at 96 h postinfection. In contrast, 229E replicated rapidly, peaking at 24 h after which the amount of infectious virus steadily declined reaching the limit of detection by 168 h postinfection. Viral cellular RNA also reduced from this time; however, it remained detectable above \((\sim 1 \log)\) input virus. This persistence of viral RNA, after infectious virus is no longer detectable, has been reported previously with other viruses such as influenza (8, 18). Currently, the diagnosis of coronavirus disease 2019 (COVID-19) relies on the detection of SARS-CoV-2 RNA using PCR, and the persistence of viral RNA has been noted, particularly in the stool (33). However, there are important limitations to molecular methods. Out of 90 RT-PCR SARS-CoV-2-positive samples, only 26 (28.9%) demonstrated viral growth in Vero cells (2). Experimental models such as the one described in this study are important in defining the relationship between duration of viral infectivity and detection of viral RNA.

Pathogenic CoVs have also demonstrated distinct individual replication kinetics. Kindler et al. (20) reported that pBEC-ALI cultures were highly permissive to MERS-CoV infection with peak virus production at 48 h postinfection, while SARS-CoV-1 replicated more slowly in comparison, peaking at 72–96 h post infection (20). More recently, Chu et al. (2020) demonstrated in ex vivo human lung tissue explants that SARS-CoV-2 produced 3.2 fold higher amounts of infectious virus particles within 48 h post-infection and significantly higher viral N antigen expression in comparison to tissues infected with SARS-CoV-1 (4). These findings might explain the high viral load in the respiratory secretions of patients with COVID-19 with mild symptoms or even during the pre-symptom incubation period (34), contributing to its transmissibility and pathogenicity.

**229E infection induces higher IFN expression compared with OC43.** A key finding of this study is that 229E infection induced significantly greater expression of IFN-\(\beta\) and IFN-\(\lambda\) in comparison to OC43. In contrast, there was variable and constitutive expression of IL-6 observed during the time course. IL-6 expression at 96 h postinfection indicated a trend for induction by 229E. This was not apparent for OC43 and presents further evidence of an impaired innate response to this virus, despite its robust replication. Our interpretation of lack of innate immune response to OC43 relates to level of RNA synthesis and threshold for innate immune activation: 229E replicated rapidly to maximize production of infectious virus. High level viral replication demands high level RNA synthesis—the cost of this is activation of innate immunity which limits duration of viral replication. In contrast, OC43 replication built up slowly—this required far less RNA synthesis at any given time which did not trigger innate immunity. This allowed OC43 to replicate productively for an extended time.

Replication without induction of an immune response has also been reported for pathogenic CoVs. This has significant implications for asymptomatic transmission, as immune response and airway inflammation is a primary driver of symptoms. Reduced innate immune response (particularly in the upper respiratory tract) and lack of associated symptoms during the initial phase of infection coupled with high viral shedding differentiates SARS-CoV-2 from SARS-CoV-1 and is almost certainly why COVID-19 has become a pandemic disease (10, 31). SARS-CoV-2 infection induced lower levels of IFNs than SARS-CoV-1 despite significantly higher viral loads in ex vivo cultures of lung biopsies (4). Another study reported that SARS-CoV-2 did not induce IFN expression at all but, in contrast to OC43, elicited a strong chemotactic and inflammatory response (including CCL20, CXCL1, IL-1B, IL-6) in human BECs (1). This lowered antiviral and heightened proinflammatory state is proposed to contribute to COVID-19. This points to OC43 as a useful virus to study treatments that aim to restore an antiviral response to SARS-CoV-2.
This study also highlights the potential for differentiated pBECs to be utilized as a platform for CoV antiviral drug development. Recent studies have focused on cell lines to investigate efficacious drugs to progress to in vivo testing. Additionally, an in vitro infection model may sustain viral replication for many days, with replication kinetics and innate immune activation more representative of that in tissues ex vivo and in vivo. This is particularly important to study OC43 and other CoVs that demonstrate cellular tropism for ciliated cells. Monolayer cultures consist only of basal cells while epithelial development. Recent studies have focused on cell lines to investigate pBECs to be utilized as a platform for CoV antiviral drug development.

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