Infection and Propagation of Human Rhinovirus C in Human Airway Epithelial Cells

Weidong Hao, Katie Bernard, Nita Patel, Nancy Ulbrandt, Hui Feng, Catherine Svabek, Susan Wilson, Christina Stracener, Kathy Wang, JoAnn Suzich, Wade Blair, and Qing Zhu

Department of Infectious Diseases and Vaccines, MedImmune LLC, Gaithersburg, Maryland, USA

Human rhinovirus species C (HRV-C) was recently discovered using molecular diagnostic techniques and is associated with lower respiratory tract disease, particularly in children. HRV-C cannot be propagated in immortalized cell lines, and currently sinus organ culture is the only system described that is permissive to HRV-C infection *ex vivo*. However, the utility of organ culture for studying HRV-C biology is limited. Here, we report that a previously described HRV-C derived from an infectious cDNA, HRV-C15, infects and propagates in fully differentiated human airway epithelial cells but not in undifferentiated cells. We demonstrate that this differentiated epithelial cell culture system supports infection and replication of a second virus generated from a cDNA clone, HRV-C11. We show that HRV-C15 virions preferentially bind fully differentiated airway epithelial cells, suggesting that the block to replication in undifferentiated cells is at the step of viral entry. Consistent with previous reports, HRV-C15 utilizes a cellular receptor other than ICAM-1 or LDLR for infection of differentiated epithelial cells. Furthermore, we demonstrate that HRV-C15 replication can be inhibited by an HRV 3C protease inhibitor (rupintrivir) but not an HRV capsid inhibitor previously under clinical development (pleconaril). The HRV-C cell culture system described here provides a powerful tool for studying the biology of HRV-C and the discovery and development of HRV-C inhibitors.

Human rhinovirus (HRV) is a nonenveloped, positive-strand RNA virus belonging to the *Picornaviridae* family and is typically categorized into three species (HRV-A, HRV-B, or HRV-C) based on phylogenetic sequence analysis (34). HRV is the most frequent etiological agent of acute upper respiratory tract infections (URIs) (28) and has been associated with severe lower respiratory tract infections (LRIs) with a spectrum of clinical outcomes (13). There is considerable clinical and epidemiologic evidence linking HRV infections to bronchitis, bronchiolitis, pneumonia, and asthma and chronic obstructive pulmonary disease (COPD) exacerbations (8, 18, 31, 46). In addition, HRV-associated wheezing in infancy is predictive of the development of childhood asthma (17). Previous reports suggested that HRV-C is associated with more severe illness than HRV-A or HRV-B (19, 32, 43, 47). More recent studies show that HRV-A as well as HRV-C are the most prevalent HRVs identified in hospitalized children (7, 27, 37, 41, 49), while HRV-C infection is associated with an increase in hospitalization events in atopic children (4). The role of HRVs in respiratory disease is currently an area of intense research, and the emerging data indicate a significant role for HRV-C in lower respiratory tract disease, particularly in children (10, 21, 22, 26, 30, 38, 48).

HRV-A and -B are comprised of ~100 serotypes and can be readily propagated in tissue culture using immortalized cell lines. HRV-A and -B can be further subdivided into major and minor groups based on cellular receptor utilization. Approximately 85% of HRV-A and 100% of HRV-B serotypes utilize intercellular adhesion molecule 1 (ICAM-1) as a receptor to enter cells and are referred to as the major group (9, 34, 44). The remaining 15% of HRV-A serotypes utilize a member of the low-density lipoprotein receptor (LDLR) family as a cellular receptor and thus are categorized as the minor group (16). Currently >60 distinct HRV-C sequences have been described using molecular diagnostic techniques (40). In contrast to HRV-A or -B, HRV-C cannot be propagated in immortalized cell lines using standard tissue culture

methods, and at least one HRV-C isolate (HRV-C15) utilizes a cellular receptor other than ICAM-1 or LDLR (2).

Multiple therapeutic approaches targeting HRV have been progressed into the clinic, including small-molecule capsid-binding inhibitors (pleconaril [11], pirodavir [1, 14], and BTA798 [3]), a 3C protease inhibitor (rupintrivir [35]), and soluble ICAM-1 (45). Although several inhibitors showed a modest antiviral effect in early clinical trials, none have been approved for treatment. In the case of pleconaril, drug resistance was observed in 24% of patients, with 13% of patients harboring pleconaril-resistant virus at baseline (11, 12). The majority of the clinical compounds were developed prior to the discovery of HRV-C, and it is unclear if any of the inhibitors exhibit antiviral activity against HRV-C strains. Given the frequency of HRV-C-associated lower respiratory tract disease, antiviral activity against HRV-C should be a critical component of HRV drug development going forward.

Efforts to propagate HRV-C in immortalized cell lines have not been successful to date (2, 22, 23, 30), therefore studying the biology of HRV-C and evaluating inhibitors has been difficult. A recent study by Bochkov et al. reported the infection of sinus mucosal organ culture with two HRV-C isolates and the generation of an infectious cDNA clone for one, HRV-C15 (2). This study represents a significant advancement toward the development of a functional HRV-C cell culture system. However, the nature of the organ culture system and the scarcity of available organ specimens limit the utility of such a system for studying the biology of HRV-C and screening for inhibitors. In this study, we describe a

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Address correspondence to Qing Zhu, ZhuQ@MedImmune.com.

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primary cell culture system that supports HRV-C infection and can be more readily utilized to study HRV-C biology as well as screen for inhibitors of HRV-C replication. We exploit air-liquid interface techniques to culture fully differentiated human airway epithelial cells. We demonstrate that HRV-C15 infects fully differentiated airway epithelial cells (nasal or bronchial epithelial) but not undifferentiated airway epithelial cells, and that HRV-C15 can be serially passaged. We describe the generation of a second infectious molecular clone of HRV-C (HRV-C11 [42]) that can be propagated in differentiated airway epithelial cells. We show that HRV-C virions bind to fully differentiated airway epithelial cells but exhibit significantly decreased binding to undifferentiated cells, suggesting that the block to replication in undifferentiated cells is at the step of virus binding/entry. Furthermore, our data show that HRV-C infection is blocked by an HRV 3C protease inhibitor (rupintrivir) but not by an HRV capsid inhibitor (pleconaril).

MATERIALS AND METHODS

Cell culture and viruses. Normal human bronchial epithelial and nasal epithelia cells were purchased from Lonza (Walkersville, MD) and PromoCell (Heidelberg, Germany), respectively. The bronchial epithelial and nasal epithelial cells were cultured under air-liquid interface (ALI) conditions to induce differentiation. Briefly, cells were grown on collagen-coated cell culture inserts (Costar, NY). Once cells reached confluence, they were exposed to the air-liquid interface and cultured for approximately 2 to 3 weeks in a medium consisting of high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with a bronchial epithelial growth medium (BEGM) bullet kit (Lonza) or a nasal airway epithelial cell growth medium kit (PromoCell) to induce mucociliary differentiation.

HeLa-H1 cells were obtained from the ATCC. Cells were cultured in DMEM supplemented with 1% penicillin-streptomycin, 1% HEPES buffer solution, and 10% heat-inactivated fetal bovine serum.

HRV-A16 and HRV-B14 viruses were purchased from the ATCC and propagated in HeLa-H1 cells, and infectious virus in the resulting viral stocks were quantitated by plaque assay as previously described (33).

Construction of HRV-C cDNAs and RNA transfection. Full-length HRV-C genomes (HRV-C15; GenBank accession no. GU219984 [2]; and HRV-C11; accession no. EU840952 [42]) were chemically synthesized based on the published sequences. The T7 promoter sequence (TAATAC GACTCACTATAGG) was added at the 5' end of each HRV-C full-length cDNA sequence (GeneWiz, NJ). To construct a replication-deficient mutant (HRV-C15-GAA), site-directed mutagenesis (Stratagene) was used to introduce GAA amino acid substitutions in sequences encoding the highly conserved GDD motif in the RNA-dependent RNA polymerase (20). HRV-A16 and HRV-B14 full-length genomes were amplified by reverse transcription-PCR (RT-PCR) from virus stocks and inserted into pUC19. The identity of each viral cDNA insert was confirmed by sequence analysis.

To generate *in vitro*-transcribed RNA, plasmid cDNAs were linearized by restriction enzyme digestion. Transcription reactions were performed using the T7 MEGascript kit (Invitrogen, CA) according to the manufacturer's instructions. *In vitro*-transcribed RNA (1.6 µg) was introduced into single-cell suspensions of HeLa-H1 cells by electroporation (capacity, 500 µF; voltage, 160 V; cuvette size, 0.2 cm; cell volume, 400 µl [4×10^7 cells per ml]) using Gene Pulser Xcell (Bio-Rad, CA). Electroporated cells were transferred to a 6-well plate and harvested at 2, 24, and 48 h after electroporation. Total RNA was extracted from electroporated cells using an RNeasy minikit (Qiagen, Germany), and HRV RNA was quantified as described below.

Quantification of HRV RNA by RT-PCR. HRV RNA was quantified using an AgPath-ID one-step RT-PCR kit (Invitrogen, CA) with the probe 6-carboxyfluorescein-TCC GGC CCC TGA ATG YGG CTA A-6-car-

boxytetramethylrhodamine and the following four primers: Forward-1, 5'-GCC YGC GTG GCT GCC-3'; Forward-2, 5'-GCC YGC GTG GTG CCC-3'; Forward-3, 5'-GCC TGC GTG GCG GCC-3'; and reverse primer, 5'-GCC TGC GTG GCG GCC-3'. These primers are complementary to the 5' nontranslated region of HRV. HRV RNA levels were normalized by comparison to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA levels in cell and tissue lysates. *In vitro*-transcribed HRV-C15 RNA was used to generate a standard curve. RT-PCRs were performed in duplicate for each sample and quantified on an ABI 7000 real-time PCR system (Applied Biosystems, CA). Data were analyzed using GraphPad Prism (GraphPad Software, Inc.).

Density gradient ultracentrifugation and Western analysis. HRV-C15 and HRV-A16 virions were purified using a 10 to 40% potassium tartrate gradient as described previously (51). Two hundred-µl fractions were collected from the top of the gradient and subjected to Western blot analysis. Briefly, proteins from the potassium tartrate gradient fractions were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred onto a nitrocellulose membrane. HRV-C VP1 protein was detected using a rabbit polyclonal antiserum raised against VP1 of HRV-C4 (Covance, PA) that recognizes VP1 proteins from HRV-C15 as well as HRV-C4 (data not shown). HRV VP1 proteins in the Western analysis were visualized using a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (111-035-144; Jackson) combined with a chemiluminescence substrate (LumiGLO; 546100; KPL).

Viral particle purification and EM sample preparation. Viral particles derived from HRV-C15-transfected HeLa cells were partially purified by centrifugation through a sucrose cushion similar to that described previously (5). Briefly, HRV-C15-transfected cells were incubated at 33°C for 48 h and then snap-frozen and thawed 3 times. Cell debris was removed by centrifugation at $8,699 \times g$ for 20 min, and the resulting supernatants were combined with 10% SDS and added dropwise on top of a 30% sucrose solution prepared with phosphate-buffered saline (PBS). Samples were then centrifuged at $284,061 \times g$ for 90 min at 16°C using the SW40 rotor (Sorvall). Viral pellets were resuspended in PBS, and viral particles in these enriched pellets were examined using negative-stain transmission electron microscopy (TEM) (NanoImaging Service, CA).

Sinus tissue infection. The fresh nasal mucosal samples were obtained from a 52-year-old Asian male less than 8 h postmortem (Analytical Biological Services, Inc., DE). All samples tested negative for hepatitis C virus, hepatitis B virus, and human immunodeficiency viruses 1 and 2. Tissue samples were cut into sections of ~4 mm by 4 mm, placed in a 24-well plate, and incubated with mock lysate or the apical washes of HRV-C15-infected nasal epithelial cells in a total volume of 0.6 ml of bronchial epithelial growth medium (BEGM). After 4 h of incubation at 33°C, the medium was aspirated and the samples were washed three times with PBS, and 0.6 ml of BEGM was added back to each well. HRV RNA was then quantified in the media of each well at 0.5, 24, 48, and 72 h postinfection. HRV RNA quantified at 0.5 h postinfection served as the baseline.

IFA. Immunofluorescent assay (IFA) was performed as described previously (39). In brief, sinus cultures were inoculated with HRV-C15 or medium and incubated for 24 h. Tissue samples were then paraffin embedded and sectioned onto slides. These slides were permeabilized with methanol, blocked with 5% bovine serum albumin (BSA), and incubated with the rabbit polyclonal anti-HRV-C VP1 polyclonal antibody described above. VP1 protein in infected cells was then visualized with a goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC; 20301; Invgenex, CA). Cell nuclei were stained with propidium iodide (PI; P3566; Invitrogen).

Infection of human airway epithelial cells. Bronchial epithelial or nasal epithelia cells were incubated for 2 h at 33°C with lysates of HRV-transfected HeLa cells that were passed through a 0.2-µm filter (Whatman, MA). After the 2-h incubation, the lysate was aspirated and each well was washed 3 times with PBS. At each time point thereafter (24 and 48 h), an apical wash was collected by applying 200 µl of media to the apical surface of the wells and incubating for 30 min at 33°C. In serial passage

experiments, the apical wash was passed through a 0.2- μ m filter and used to infect fresh nasal epithelial cells. To quantify HRV RNA in infected cells, cells were lysed with RLT buffer (Qiagen) supplemented with 40 μ M dithiothreitol (Thermo Fisher). RNA was extracted using the RNeasy minikit and resuspended in 100 μ l water for subsequent RT-PCR as described above.

Virus binding assay. Virus binding assays were performed using a protocol similar to that described previously (15, 39). In brief, bronchial epithelial cells cultured in transwell plates were dissociated using Stem-Pro Accutase (Invitrogen, CA) and spotted on microscope slides. Alternatively, HeLa cells were plated directly on microscope slides and incubated overnight. Cells were then fixed using 3% paraformaldehyde (PFA). Following fixation, slides were gently washed 3 times with PBS. Lysates of HRV-C15 RNA or mock-transfected HeLa cells were added to the fixed slides and incubated for 30 min at 33°C. Following the incubation, slides were washed 3 times with PBS–0.001% Tween 20 and fixed again in 3% PFA. The slides were then blocked for 1 h with 1% BSA at 37°C and incubated with rabbit polyclonal anti-HRV-C VP1. VP1 protein-antibody complexes bound to cells were visualized using an Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen). The nucleus was stained with Prolong Gold with 4',6-diamidino-2-phenylindole (DAPI) mounting media (Invitrogen). Virion binding was quantified by measuring fluorescent signal intensity per unit area for 10 randomly chosen cells using NIH ImageJ software (<http://rsb.info.nih.gov/ij/>). Virion binding to differentiated cells was compared to that to undifferentiated cells using a Student's *t* test to determine if the observed differences were significant. In addition, HRV-B14 binding to differentiated or undifferentiated cells was compared to that of the respective mock control using a Student's *t* test to determine if the observed binding was significant.

Inhibition assays. Differentiated bronchial epithelial cells were washed with PBS to disrupt any mucus on the apical surface. To evaluate the potential antiviral activity of the anti-ICAM-1 monoclonal antibody (6), cells were preincubated with an anti-ICAM-1 antibody (6) prior to addition of virus to the wells. To evaluate the potential antiviral activity of the small-molecule inhibitors or soluble LDLR (sLDLR; R&D Systems, MN), virus was preincubated with the capsid-binding inhibitor pleconaril (Acme Bioscience, Inc., CA), 3C protease inhibitor rupintrivir (Santa Cruz Biotechnology, CA), or sLDLR for 30 min at 33°C, and then virus and compound were added to differentiated epithelial cultures. After incubation for 30 min at 33°C, infected cells were washed with PBS, and growth medium containing antibody, small-molecule inhibitors, or soluble LDLR was added back to the cells. After 48 h of incubation at 33°C, cells were washed and lysed, and total RNA was extracted for RNA quantification. Percent inhibition was determined by comparing RNA levels in infected cells in the presence of inhibitor to that observed in infected cells in the presence of either an IgG1 control antibody or dimethylsulfoxide (DMSO) alone at the same concentration used in the small-molecule inhibitor incubations (0.4% final concentration).

RESULTS

Replication of transfected HRV-C RNA in HeLa cells. A recent study reported the replication of an HRV-C virus derived from a full-length cDNA clone (HRV-C15) in mucosal tissue (2). Our goal was to extend these observations by demonstrating replication of HRV-C15 in a primary tissue culture system that is amenable to inhibitor screening. To that aim, we chemically synthesized the HRV-C15 cDNA based on the published sequence (2) and introduced the HRV-C15 cDNA into a plasmid vector along with the T7 promoter. Full-length HRV-C15 RNAs were transcribed *in vitro* and transfected into HeLa cells, and RNA replication was monitored using quantitative RT-PCR (qRT-PCR) as described in Materials and Methods. HRV-C15 RNA replication was compared to that of HRV-A16 (25) RNA transfected in parallel. As shown in Fig. 1, a 116-fold increase in HRV-C15 RNA was

FIG 1 HRV RNA replication in transfected HeLa cells. HeLa cells were transfected with *in vitro*-transcribed HRV-C15 (diamond), HRV-A16 (open square), or HRV-C15-GAA (closed square) RNAs or HRV-A16 RNA in the presence of anti-ICAM-1 MAb (triangle). Viral RNA replication was measured by qRT-PCR in cell lysates at the indicated times posttransfection. Viral RNA values were normalized to GAPDH RNA levels as described in Materials and Methods. HRV RNA levels are presented as copies per ml of cell lysate, and the values represent means $\pm$ standard deviations (SD) from three independent experiments.

measured between 8 and 32 h after transfection, followed by a slow decline in steady-state levels of RNA. An increase in RNA levels was not observed when cells were transfected with HRV-C15 RNA containing amino acid substitutions in three-dimensional sequences known to disrupt RNA polymerase activity [HRV-C15-GAA] (20) (Fig. 1). These data demonstrate that the increase in RNA levels measured in HRV-C15-transfected HeLa cells resulted from bona fide RNA replication. A similar 114-fold increase in HRV-A16 RNA was observed between 8 and 32 h after transfection; however, in contrast to HRV-C15, HRV-A16 RNA replication continued to increase throughout the 72-h experiment (Fig. 1). Since HeLa cells are permissive to HRV-A16 infection, we reasoned that the increase in HRV-A16 RNA replication observed after 32 h resulted from a spreading infection in the HeLa cell cultures. To demonstrate this, we transfected HeLa cells with HRV-A16 RNA and cultured them in the presence of an anti-ICAM-1 monoclonal antibody (MAb) (6), which prevents HRV-A16 infection. The data showed that the HRV-A16 RNA replication profile in HeLa cells in the presence of the anti-ICAM-1 MAb mirrored that of HRV-C15 (Fig. 1). These data are consistent with the hypothesis that the increase in HRV-A16 RNA replication 32 h postinfection resulted from a spreading infection of the HeLa cell culture. Furthermore, these data suggest that any virions generated in cells transfected with HRV-C15 RNA are not capable of infecting and propagating in HeLa cells.

Cells transfected with HRV-C15 RNA produce viral particles. To determine if HeLa cells transfected with HRV-C15 RNA produce viral particles, lysates of HRV-C15-transfected HeLa cells were subjected to potassium tartrate density gradient ultracentrifugation (10 to 40%) followed by Western blot analysis using a polyclonal antibody directed against highly conserved sequences in HRV-C4 VP1 (22). We demonstrated that the anti-HRV-C4 VP1 polyclonal antibody cross-reacts with HRV-C15 VP1 (data not shown). As shown in Fig. 2A, protein sedimenting to approximately the middle of the potassium tartrate gradient (fractions 33 to 39) was detected by Western analysis that exhibited the expected molecular mass for VP1 of HRV-C15 (32 kDa). When HRV-A16 virions were subjected to potassium tartrate density gradient ultracentrifugation in parallel experiments, VP1 protein was detected in similar gradient fractions (data not shown),

FIG 2 Characterization of HRV-C15 viral particles produced after transfection of HeLa cells with HRV-C15 RNA. (A) Lysates of HRV-C15 RNA-transfected HeLa cells were subjected to potassium tartrate density gradient (10 to 40%) ultracentrifugation, and the presence of HRV-C15 VP1 protein in gradient fractions was detected by Western blot analysis using a polyclonal antibody directed against HRV-C VP1. Fraction numbers are indicated and are oriented from top to bottom of the gradient. Purified HRV-C VP1 (VP1) protein was included in the Western analysis for reference. M indicates the molecular size marker. (B) Electron microscopy (EM) images of sucrose cushion-enriched HRV-C15 viral particles or a mock control. Arrows indicate viral particles (triangle head) or empty particles (diamond head). For magnification of 110,000 $\times$, the scale bar is 100 nm; for magnification of 52,000 $\times$, the scale bar is 200 nm.

strongly suggesting that VP1 containing virion particles was produced in HRV-C15-transfected HeLa cells.

To demonstrate conclusively that viral particles were produced in HRV-C15-transfected cells, lysates of HRV-C15- or mock-transfected HeLa cells were subjected to ultracentrifugation through a 30% sucrose cushion, and the resulting pellets were analyzed by electron microscopy (EM). Consistent with previous data (2), intact HRV-C15 virions as well as empty capsids approximately 30 nm in diameter were observed in the lysates of HRV-C15 RNA-transfected cells but not in the lysates of mock-transfected cells (Fig. 2B). These data demonstrate that viral particles were generated in HeLa cells transfected with HRV-C15 full-length RNAs.

HRV-C15 replicates in sinus tissue. To determine if the viral particles produced after transfection of HeLa cells with HRV-C15 RNA were infectious, sinus tissue was incubated with the lysate of HRV-C15 RNA or mock-transfected cells. RNA replication in infected cells was measured using qRT-PCR, and VP1 capsid protein expression was monitored using an immunofluorescence assay

(IFA). HRV-C15 RNA levels increased 460-fold between 4 and 48 h after incubation with lysate from HRV-C15-transfected cells (Fig. 3A). As expected, HRV RNA was not detected in tissue incubated with the lysate of mock-transfected cells (data not shown). Consistent with these observations, VP1 protein expression was detected in tissue incubated with lysate of HRV-C15-transfected cells but not mock-transfected cells (Fig. 3B). HRV-C15-infected cells were localized primarily on the outer epithelial layer of the mucosal tissue (Fig. 3B). These data reproduce previously published results (2) and show that virions capable of infecting sinus organ culture are produced in HeLa cells transfected with HRV-C15 RNA.

HRV-C infection and propagation in differentiated human airway epithelial cells. In an attempt to develop an HRV-C infection system with broad utility, we evaluated HRV-C replication in primary human nasal epithelial cells using qRT-PCR. As a control, HRV-A16 replication was also measured. Lysates of HeLa cells transfected with HRV-C15 or HRV-A16 RNAs were incubated with either undifferentiated primary nasal epithelial cells or fully

FIG 3 HRV-C15 infection of sinus tissue. (A) Sinus tissue was infected with the lysates of HRV-C15 RNA or mock-transfected HeLa cells, and viral RNA replication was measured using qRT-PCR at the indicated times after infection. HRV RNA levels are presented as copies per ml of medium collected. (B) Evaluation of VP1 protein expression in sinus tissue infected with HRV-C15 or mock infected. HRV-C15 or mock-infected tissue samples were fixed and permeabilized 24 h after infection as described in Materials and Methods, and VP1 protein expression was detected in cells using an anti-HRV-C VP1 rabbit polyclonal antibody combined with a goat anti-rabbit IgG conjugated with FITC (green). Propidium iodide was used to stain the nucleus (red).

differentiated nasal epithelial cells grown under air-liquid interface (ALI) conditions. The fully differentiated nasal epithelial cells exhibited a pseudostratified, mucociliary phenotype with abundant cilia and mucus secretion (data not shown). HRV-A16 RNA replication was detected by qRT-PCR in both undifferentiated and differentiated nasal epithelial cells with peak RNA levels achieved 24 h after infection (Fig. 4A and B). Similar results were observed for HRV-B14 (data not shown). In contrast, HRV-C15 replication was detected in differentiated nasal epithelial cells (Fig. 4B) but not undifferentiated cells (Fig. 4A). To verify HRV-C15 infection and RNA replication in differentiated nasal epithelial cells, we subjected the HRV RNA recovered from HRV-C15-infected cells to sequence analysis. The sequence analysis confirmed the identity of the HRV-C15 RNA, thus ruling out the possibility that the RNA replication measured after HRV-C15 infection was due to contaminated HRV (data not shown). To definitively demonstrate viral propagation, HRV-C15 was serially passaged in differentiated nasal epithelial cells. Cells were infected with HRV-C15 and unbound virus removed by washing, and infected cells were incubated for 48 h. An apical wash was then collected from the HRV-C15-infected cells, passed through a 0.2- μ m filter, and used to infect a separate culture of differentiated nasal epithelial cells. As shown in Fig. 4C, HRV-C15 RNA replication was detected by qRT-PCR in the newly infected nasal epithelial cells with peak RNA levels measured 48 h after infection. These data demonstrate further that HRV-C15 virions are fully infectious and can be serially passaged in culture. In addition to nasal epithelial cells, we evaluated HRV-C15 replication in differentiated human bronchial epithelial cells cultured using ALI methods. Lysates of HRV-

FIG 4 HRV infection and propagation in primary nasal or bronchial epithelial cells. (A) HRV infection of undifferentiated primary nasal epithelial cells. Lysates of HRV-C15 (diamond) or HRV-A16 (open square) RNA-transfected HeLa cells were incubated with undifferentiated nasal epithelial cells, and HRV RNA replication was monitored by qRT-PCR at the indicated times. (B) Lysates of HRV-C15 or HRV-A16 RNA-transfected HeLa cells were incubated with differentiated nasal epithelial cells, and HRV RNA replication was measured as described for panel A. (C) Propagation of HRV-C15 in differentiated nasal epithelial cell cultures. Cell-free HRV-C15 viral particles were collected from an apical wash of infected differentiated nasal epithelial cells and used to infect separate naïve cultures of differentiated nasal epithelial cells as described in Materials and Methods. Viral RNA replication was measured by qRT-PCR at the indicated times after infection. (D) Infection of differentiated human bronchial epithelial cells. Lysates of HRV-C11 (closed circle)- or HRV-C15 (diamond)-transfected HeLa cells were incubated with differentiated bronchial epithelial cells, and HRV RNA replication was measured as described for panel A.

C15-transfected HeLa cells were incubated with fully differentiated bronchial epithelial cells, and HRV-C15 RNA replication was detected using qRT-PCR. Similar to the replication kinetics observed for nasal epithelial cells, the results showed that HRV-C15 replication in differentiated bronchial epithelial cells peaked 24 h after infection (Fig. 4D). These data demonstrate that either differentiated nasal or bronchial epithelial cells are permissive to HRV-C infection.

To confirm that this primary cell culture system would support the replication of additional HRV-C isolates, we evaluated the replication of a second HRV-C derived from a cDNA clone (HRV-C11). We chemically synthesized a full-length HRV-C11 cDNA based on the published sequence (42) and transfected full-length RNAs derived from the HRV-C11 cDNA into HeLa cells. HRV-C11 exhibited RNA replication levels in transfected HeLa cells that were similar to those observed for HRV-C15 (data not shown). Lysates of HRV-C11-transfected HeLa cells were then incubated with differentiated bronchial epithelial cells, and RNA replication was measured by qRT-PCR. HRV-C11 RNA replication was observed with peak RNA levels occurring 24 h after infection (Fig. 4D). These data indicate that HRV-C11 exhibits replication kinetics comparable to those of HRV-C15 and that the differentiated bronchial epithelial cell system supports infection and replication of at least 2 different HRV-C isolates.

HRV-C15 virions bind differentiated nasal epithelial cells.

To investigate the basis for the cell specificity of HRV-C15 replication, we evaluated the binding of HRV-C15 virions to differentiated versus undifferentiated bronchial epithelial cells. Binding of HRV-B14 was evaluated in parallel as a positive control. Differentiated or undifferentiated bronchial epithelial cells were fixed and incubated with the lysates of HRV-C15, HRV-B14, or mock-transfected HeLa cells, and virion binding was monitored by fluorescence microscopy using an antibody directed against HRV VP1 capsid protein (Fig. 5A to F). The level of virion binding was quantified by measuring mean fluorescent signal intensity per unit area (in arbitrary units [AU]) using ImageJ software (Fig. 5G). Consistent with the infection results, the level of HRV-C15 virion binding detected for differentiated bronchial epithelial cells (Fig. 5F) was significantly higher than that observed for undifferentiated cells (Fig. 5E and G) ($P < 0.001$). In contrast, similar levels of HRV-B14 binding were visualized by fluorescence microscopy in both undifferentiated and differentiated cells (Fig. 5C and D). Levels of HRV-B14 binding to undifferentiated or differentiated epithelial cells were significant compared to those of the relevant mock control ($P < 0.001$) (Fig. 5G). These data are consistent with the observation that both undifferentiated and differentiated cells are permissive to infection by HRV-B14 (24). As expected, minimal binding signal (i.e., background) was detected when lysates of mock-transfected cells were incubated with undifferentiated or differentiated epithelial cells (Fig. 5A and B) or when HRV-C15 virion containing lysate was incubated with HeLa cells (data not shown). Taken together, these data show that the block to HRV-C15 infection in undifferentiated epithelial cells or HeLa cells is at least in part at the step of virion binding.

HRV-C15 utilizes a receptor other than ICAM-1 or LDLR to infect epithelial cells. To determine if HRV-C15 utilizes ICAM-1 or LDLR as a cellular receptor, differentiated bronchial epithelial cells were infected with HRV-C15, HRV-A16, or HRV-A2 in the presence of either a monoclonal antibody directed against ICAM-1 (6) or a soluble LDLR decoy (sLDLR) (29). HRV repli-

FIG 5 HRV virion binding to bronchial epithelial cells. Undifferentiated or differentiated bronchial epithelial cells were fixed on microscope slides and incubated with lysates of mock (A and B)-, HRV-B14 RNA (C and D)-, or HRV-C15 RNA (E and F)-transfected HeLa cells for 30 min at 33°C. VP1 protein on the surface of cells was detected using polyclonal anti-VP1 antibody and visualized using a fluorescently labeled goat anti-rabbit IgG (red) as described in Materials and Methods. (G) Virion binding to undifferentiated (gray bars) or differentiated bronchial epithelial cells (black bars) was quantified by measuring fluorescent signal intensity per unit area for 10 randomly chosen cells using the NIH ImageJ software and is expressed as 10^3 arbitrary units (AU). Error bars indicate SEM, and P values were calculated with Student's t test (***, $P < 0.001$; NS, not significant [$P > 0.05$]). Nuclei (blue) were stained with Prolong Gold with DAPI mounting medium.

cation was then monitored by qRT-PCR. As expected, HRV-A16 (major group) infection was blocked by the anti-ICAM-1 MAb but not by sLDLR, while HRV-A2 (minor group) infection was inhibited by sLDLR but not by anti-ICAM-1 (Fig. 6A). In contrast, HRV-C15 replication was not blocked by anti-ICAM-1 at the highest concentration tested (2 μ g/ml), and minimal inhibition (<25% inhibition) was observed in the presence of 50 μ g/ml of sLDLR (Fig. 6A). These data are consistent with a previous report (2) and demonstrate that HRV-C15 utilizes a receptor other than ICAM-1 or LDLR to enter differentiated bronchial epithelial cells.

FIG 6 HRV infection of bronchial epithelial cells in the presence of inhibitors. (A) HRV infection of bronchial epithelial cells in the presence of an anti-ICAM-1 MAb or sLDLR. Lysates of HRV-C15, HRV-A2, or HRV-A16 RNA-transfected HeLa cells were incubated with differentiated bronchial epithelial cells in the presence or absence of an anti-ICAM MAb or sLDLR, and HRV RNA replication was measured by qRT-PCR as described in Materials and Methods. Data are presented as mean ($\pm$ SD) percent inhibition of RNA replication in the presence of inhibitor relative to that observed in the presence of a control human IgG1. (B) HRV infection of bronchial epithelial cells in the presence of a 3C protease or capsid inhibitor. Lysates of HRV-C15- or HRV-B14-transfected HeLa cells were incubated with differentiated bronchial epithelial cells in the presence or absence of rupintrivir (final concentration, 500 nM) or pleconaril (final concentration, 10 μ M), and HRV RNA replication was measured by qRT-PCR as described in Materials and Methods. Data are presented as mean ($\pm$ SD) percent inhibition of RNA replication in the presence of inhibitor relative to that observed in the presence of DMSO alone.

HRV-C15 replication is blocked by a 3C protease inhibitor but not an HRV capsid inhibitor. To examine the susceptibility of HRV-C to known HRV inhibitors, we evaluated two small molecules directed at different HRV targets (3C protease or capsid). Differentiated bronchial epithelial cells were incubated with the lysates of HRV-C15- or HRV-B14-transfected HeLa cells in the presence or absence of different concentrations of a 3C protease inhibitor (rupintrivir) (35) or an HRV capsid inhibitor (pleconaril) (36). In the presence of 500 nM rupintrivir, HRV-C15 replication was inhibited >99% (Fig. 6B). Subsequent concentration-response assays showed that rupintrivir inhibited HRV-C15 replication with a mean 50% effective concentration (EC_{50}) of 93 nM, which was comparable to that observed for other picornaviruses (35). Alternatively, pleconaril exhibited minimal levels of HRV-C15 inhibition (<5%) at high concentrations (10 μ M) (Fig. 6B). As expected, both rupintrivir (500 nM) and pleconaril (10 μ M) suppressed replication of HRV-B14 by >98% (Fig. 6B).

DISCUSSION

In this study, we describe a primary cell culture system that supports the replication of 2 different HRV-C viruses. We demonstrate RNA replication, viral protein expression, and viral particle formation in HeLa cells after transfection with full-length HRV-C15 RNA. The resulting HRV-C15 viral particles can infect sinus tissue and fully differentiated human nasal or bronchial epithelial

cells. Bona fide HRV-C15 propagation was demonstrated by serial passage of virus in differentiated nasal epithelial cell cultures. We show that HRV-C15 replication can be inhibited by an HRV 3C protease inhibitor, further validating that the observed RNA replication is dependent on viral protein expression and function. The methodology described here enables future studies characterizing the replication of HRV-C and virus-host cell interactions.

Previous attempts to propagate HRV-C isolates in cell lines or primary cell culture were not successful (2, 22, 23, 30). The successful propagation of HRV-C in our study was dependent on culturing nasal or bronchial epithelial cells under air-liquid interface conditions that favored the development of a mucociliary epithelium resembling native epithelium in both structure (full confluence, formation of tight junctions and cilia) and function (electrical resistance and mucus secretion). This is in contrast to that observed for HRV-A16 or HRV-B14, which replicated efficiently in undifferentiated as well as differentiated airway epithelial cells. These results appear analogous to those reported for respiratory syncytial virus (RSV) (50) and suggest that both HRV-C and RSV preferentially infect and replicate in ciliated cells in the airway. However, we did not directly demonstrate HRV-C replication in ciliated cells, and it is possible that both ciliated and nonciliated cells support HRV-C replication in differentiated airway epithelial cultures. In fact, HRV-C15 infection was previously reported in both nonciliated and ciliated cells in sinus organ culture (2). A more detailed analysis of the specific cell types that support HRV-C replication in airway epithelial cells can be addressed in future studies.

To expand the utility of the HRV-C cell culture system, we generated a second HRV-C infectious clone (HRV-C11) and demonstrated HRV-C11 replication in differentiated epithelial cells. Although HRV-C15 and HRV-C11 are related, there is a moderate degree of intrasubtype diversity (31% amino acid sequence divergence) based on phylogenetic analysis (42). The availability of two separate HRV-C infectious clones will further enable studies on HRV-C biology and inhibitor screening. It is reasonable to assume with an increased level of confidence that observations common to both HRV-C11 and HRV-C15 are generalizable to other members of the HRV-C subtype compared to phenomena observed for a single HRV-C clone. In addition, our results suggest that differentiated airway epithelial cells support the replication of other HRV-C isolates such that a panel of infectious HRV-C viruses might be constructed in the future.

Although RNA replication and HRV-C infectious virus production were readily detected in HeLa cells after transfection of HRV-C15- or HRV-C11-derived RNAs, we and others were not able to detect virus propagation in HRV-C15-transfected HeLa cells or infection of HeLa cells exposed to HRV-C15 virions (2). HRV-C15 virion binding to HeLa cells was not detected. These data strongly suggest that the block to HRV-C infection of HeLa cells is at the step of viral entry. In addition, we were able to detect HRV-C virion binding to differentiated airway epithelial cells, which were permissive to infection, but not undifferentiated cells. Consistent with the observations in HeLa cells, these results indicate that the block to infection in undifferentiated epithelial cells is also at the step of virion binding/entry. ICAM-1 and members of the LDLR family are utilized as cellular receptors by the major and minor HRV-A and HRV-B, respectively. However, our data and data from a previous study (2) showed that HRV-C15 infection is not dependent on ICAM-1 or LDLR. One attractive hypothesis is

that HRV-C utilizes a distinct receptor for cell entry and that nasal or bronchial epithelial cell differentiation is required for HRV-C receptor expression. Such observations suggest that a comparison of molecules expressed on the cell surface of differentiated versus undifferentiated nasal or bronchial epithelial cells is a strategy to identify the HRV-C receptor(s).

One of the goals for this study was to establish a robust primary cell culture system that can be used to evaluate the antiviral activity of potential HRV-C inhibitors. To illustrate the utility of the system, we evaluated the antiviral activity of two known HRV inhibitors (rupintrivir and pleconaril). Our data showed that HRV-C15 was sensitive to inhibition by rupintrivir with an EC₅₀ comparable to that observed for other picornaviruses (35). However, suppression of viral replication was not observed when cells were infected with HRV-C15 in the presence of pleconaril even at high concentrations (10 μ M). Our results call into question the potential therapeutic utility of pleconaril or possibly other related capsid inhibitors in the treatment of HRV-C infection. In a previous study, HRV-C15 infection of sinus organ culture was inhibited by WIN-56291, a small molecule structurally related to pleconaril. It is possible that small differences in chemical structure lead to differences in antiviral activity; however, the structure-activity relationship (SAR) of targeting HRV-C capsid will need to be investigated further. In addition, it remains to be determined if pleconaril exhibits antiviral activity against HRV-C isolates other than HRV-C15. Either way, these results highlight the importance of careful evaluation of the activity of HRV inhibitors in an HRV-C cell culture system to begin to understand their potential therapeutic utility in HRV-C respiratory disease.

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