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Respiratory Syncytial Virus Infection of Human Airway Epithelial Cells Is Polarized, Specific to Ciliated Cells, and without Obvious Cytopathology

Liqun Zhang,[1]* Mark E. Peeples,[2] Richard C. Boucher,[1] Peter L. Collins,[3] and Raymond J. Pickles[1]

Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina, Chapel Hill, North Carolina 27599-7248[1]; Department of Immunology/Microbiology, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612[2]; and Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-0720[3]

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Gene therapy for cystic fibrosis (CF) lung disease requires efficient gene transfer to airway epithelial cells after intralumenal delivery. Most gene transfer vectors so far tested have not provided the efficiency required. Although human respiratory syncytial virus (RSV), a common respiratory virus, is known to infect the respiratory epithelium, the mechanism of infection and the epithelial cell type targeted by RSV have not been determined. We have utilized human primary airway epithelial cell cultures that generate a well-differentiated pseudostratified mucociliary epithelium to investigate whether RSV infects airway epithelium via the lumenal (apical) surface. A recombinant RSV expressing green fluorescent protein (rgRSV) infected epithelial cell cultures with high gene transfer efficiency when applied to the apical surface but not after basolateral inoculation. Analyses of the cell types infected by RSV revealed that lumenal columnar cells, specifically ciliated epithelial cells, were targeted by RSV and that cultures became susceptible to infection as they differentiated into a ciliated phenotype. In addition to infection of ciliated cells via the apical membrane, RSV was shed exclusively from the apical surface and spread to neighboring ciliated cells by the motion of the cilial beat. Gross histological examination of cultures infected with RSV revealed no evidence of obvious cytopathology, suggesting that RSV infection in the absence of an immune response can be tolerated for >3 months. Therefore, rgRSV efficiently transduced the airway epithelium via the lumenal surface and specifically targeted ciliated airway epithelial cells. Since rgRSV appears to breach the lumenal barriers encountered by other gene transfer vectors in the airway, this virus may be a good candidate for the development of a gene transfer vector for CF lung disease.

Human respiratory syncytial virus (RSV) is the most important viral agent causing serious pediatric respiratory disease worldwide (5). RSV infection causes common-cold-like symptoms that progress to lower respiratory tract disease in 25 to 40% of infected infants and results in hospitalization for 0.1 to 1.0% of those infected. Almost everyone has been infected by RSV by 2 years of age. The immunity induced by RSV infection typically is incomplete, and reinfection is common, although subsequent infections are partially restricted and the disease severity is reduced (5).

There is a relative lack of direct, detailed information on the characteristics of RSV infection, spread, and pathology in the upper and lower respiratory tract of humans. RSV infects the superficial layer of the respiratory epithelium and has the capacity to spread throughout the conducting airways. However, the details of RSV infection remain unclear, including whether there is a cell specificity to infection, the extent of virus infection and its relationship to disease manifestation and severity, the extent and cause of tissue damage, and whether the formation of syncytia that are so prominent in nonpolarized cells is important for the pathogenesis observed in vivo.

* Corresponding author. Mailing address: Cystic Fibrosis Center, University of North Carolina, 7129 Thurston-Bowles, CB 7248, Chapel Hill, NC 27599-7248. Phone: (919) 966-7044. Fax: (919) 966-7524. E-mail: liqun_zhang@med.unc.edu.

RSV is an enveloped, nonsegmented, negative-sense RNA virus classified in the subfamily *Pneumovirinae* of the family *Paramyxoviridae*. When propagated in established cell lines, RSV has been visualized as pleomorphic spheres 120 to 300 nm in diameter and, more frequently, as long filaments of up to 1 to 10 μm in length (5, 34). RSV replicates relatively inefficiently in vitro, and most of the progeny virus remain cell associated (5, 22). In contrast, RSV replicates to relatively high titer in the respiratory tract of a permissive host, such as chimpanzee and human (5). Like those of other members of the family *Paramyxoviridae*, RSV gene expression and replication appear to be entirely cytoplasmic, with no apparent direct nuclear involvement. The 15.2-kb RNA genome has been completely sequenced and has been shown to contain 10 mRNAs encoding 11 distinct proteins, and one or more functions have been identified for most of the viral proteins. Complete infectious recombinant virus has been rescued from plasmids encoding a complete positive-sense copy of the genome together with the proteins of the nucleocapsid-polymerase complex, namely, the nucleocapsid (N) protein, phosphoprotein (P), large polymerase protein (L), and transcription antitermination factor (M2-1) (6).

RSV encodes three virion surface proteins: the heavily glycosylated G protein, which was previously identified as an attachment protein (23); the fusion (F) protein, which mediates membrane fusion at the cell surface, resulting in viral

penetration; and the SH protein, which does not appear to be necessary for any step in the virus replicative cycle and whose function remains unknown. An RSV variant called cp-52 that was derived by extensive passage of wild-type virus in vitro was shown to lack the SH and G genes due to a spontaneous deletion (18), and recombinant viruses have been developed that lack the G gene, the SH gene, or both (4, 36). The ability of these G deletion RSV mutants to replicate efficiently in cultured cells (18, 36) indicated that the G protein, like SH, is also dispensable for infection, syncytium formation, and virion morphogenesis, at least in vitro. This observation suggested that the F protein, the sole remaining viral surface protein, can also act as an attachment protein (3, 36). The RSV envelope protein-target cell interaction has been studied predominately in nonpolarized immortal epithelial-cell-like cell lines. Recent studies have suggested that sulfated glycosaminoglycans on the membranes of such cell lines are involved in RSV infection (12, 13, 21, 24). Both the G and F proteins have been shown to bind to heparin, a model glycosaminoglycan (9), consistent with the idea that each protein might function in attachment. In polarized Vero cell monolayers, RSV infection was shown to result in the budding and release of virus from the apical surface (34).

In the present study, we characterized RSV infection of human airway epithelium (HAE) cells using a model of well-differentiated (WD) HAE. WD HAE cultures are derived directly from HAE tissue and, following seeding in vitro, grow to establish a multilayered, polarized, and differentiated tissue culture that closely resembles the airway epithelium in vivo with regard to morphology and functions, including mucus production and ciliary motion (25, 31). RSV infection was performed using a recombinant RSV that expresses green fluorescent protein (GFP) (rgRSV), providing the means to visualize infection of living cells (36). Our studies show that RSV preferentially targets the ciliated cells of the airway epithelium and that infection (and subsequent virus release) occurs exclusively via the apical surface. In addition, RSV infection persists in this tissue model for >1 month without obvious cytopathic effects at the light microscopic level, whereas in contrast, infection with influenza A virus results in rapid and extensive obvious cytopathology.

MATERIALS AND METHODS

Viruses. The construction of rgRSV (224) has been described in detail elsewhere (13). Briefly, GFP (Life Technologies, Gaithersburg, Md.) was engineered to be flanked by RSV gene start and gene end sequences and was inserted as the first, promoter-proximal gene in a full-length cDNA of the wild-type RSV strain A2 antigenomic RNA. rgRSV was rescued by cotransfecting HEp-2 cells with the antigenomic plasmid and N, P, M2-1, and L support plasmids and infecting them with a modified vaccinia virus, MVA-T7, expressing T7 RNA polymerase (42). Virus stocks were prepared in HEp-2 cells and were aliquoted and stored at −80°C until use. For HEp-2 cells, rgRSV was found to replicate to near-parental titers and to produce syncytia at a rate similar to that of the parental virus. Recombinant wild-type RSV without GFP (GP1) and a biologically derived RSV strain (Hep-4) were also used in this study. The Udorn strain of influenza A virus (A/Udorn/72) was provided by Brian Murphy (National Institute of Allergy and Infectious Disease) and was propagated in HEp-2 cells in the presence of 0.75 μg of trypsin/ml. Nonreplicating adenoviral vectors expressing GFP (AdVGFP) were obtained from the University of North Carolina Gene Therapy Vector Core Facility.

Viruses released into the apical compartment were harvested by adding 200 μl of medium to the apical surface of the culture for 20 min and, after retrieval with a pipette, combining it with an equal volume of 2× viral stabilizing solution (200 mM $MgSO_4$, 100 mM HEPES, pH 7.5), snap freezing the mixture on dry ice, and storing it at −80°C. Two hundred microliters of basolateral medium from a total of 1 ml was retrieved and combined with an equal volume of 2× viral stabilizing solution as described above. Viral titration was performed as described previously and corrected for differences in sample volume (27).

WD HAE cell culture. Human nasal, tracheobronchial, and bronchiolar airway epithelial cells were obtained from cystic fibrosis (CF) patients and non-CF patients undergoing surgical procedures, and epithelial cells were isolated by the University of North Carolina Cystic Fibrosis Center Tissue Culture Core Facility using Institutional Review Board-approved protocols. Following enzymatic dispersion, cells were seeded on collagen-coated, semipermeable membrane supports (Transwell-Col; 12 mm in diameter; 0.4-μm pore size; Corning-Costar, Corning, N.Y.) as previously described (31). At confluence, the apical medium was removed and the cells were maintained at an air-liquid interface (ALI) to allow differentiation of the epithelial subtypes. WD cultures, identified as cultures containing ciliated cells and with transepithelial resistances of ≥300 $\Omega \cdot cm^2$, were studied approximately 4 to 6 weeks after initiation of an ALI unless otherwise stated. In some cases, primary cells were further expanded on tissue culture dishes before being seeded onto Transwells (passage 1 cells). Both primary and passage 1 cultures derived from non-CF and CF sources were used in the study and showed no significant differences in any of the parameters tested.

Viral inoculation of HAE cultures. Frozen aliquots of rgRSV or AdVGFP were thawed immediately before use and diluted in tissue culture medium. After the apical surfaces of HAE cultures were rinsed with medium, 100 μl of viral suspension was applied to the apical surface for 1 h at 37°C, and the virus was removed by washing with medium. Inoculation of the basolateral surface of the cultures was performed by inverting the insert and exposing the permeable support to a volume and a concentration of virus equal to those used for the apical inoculation.

For experiments with RSV antibody and ribavirin, the reagents were diluted in tissue culture medium immediately before use. Antibody (250 μg/ml) was applied to the apical or basolateral surfaces of cultures at the time of rgRSV inoculation or as otherwise noted. Ribavirin was added to the basolateral medium at a final concentration of 100 μg/ml.

Photomicrographs of GFP-expressing cells were acquired using a Leica Leitz DM IRB fluorescence inverted microscope equipped with a Hamamatsu C5810 color 3 chilled charge-coupled device digital camera and Adobe Photoshop. Quantitation of infected cells was performed with the image-processing toolkit plug-ins for Photoshop (ISBN 1-928808-00-X).

Immunolocalization of ciliated-cell-specific KS. Keratan sulfate (KS) immunolocalization was performed with HAE cultures fixed with 4% paraformaldehyde. The apical surfaces of cultures were directly exposed to 10% normal goat serum to block nonspecific attachment prior to addition of a KS-specific monoclonal mouse immunoglobulin G (IgG) antibody (MAB2022; Chemicon, Temecula, Calif.), followed by goat anti-mouse IgG-conjugated to Texas Red (Jackson ImmunoResearch, West Grove, Pa.). Texas Red fluorescence was recorded by optical sections using confocal laser scanning microscopy (Leica DM IRBE).

Reagents. Humanized monoclonal antibody directed to the F protein of RSV (Synagis) was obtained as a kind gift from MedImmune Inc. (Gaithersburg, Md.). Ribavirin was purchased from ICN Biochemicals Inc. (Aurora, Ohio). All other reagents and chemicals, unless otherwise noted, were obtained from Sigma Chemical Company (St. Louis, Mo.).

RESULTS

Polarity of rgRSV infection in HAE cultures. We previously used rgRSV to monitor infection of HEp-2 cell monolayers, in particular to characterize the involvement of cell surface glycosaminoglycans in virus attachment and infection in vitro (12, 13). In the present study, we used rgRSV to monitor infection of WD HAE cultures. These cultures are polarized and pseudostratified, with mucociliary cells at the apical surface, similar in both morphology and cell type distribution to the respiratory epithelium in vivo. The pseudostratified mucociliary epithelial cultures are composed of a number of different cell types, including lumen-facing ciliated cells, mucus-secreting cells, and intermediate and basal cell types in the basolateral compartment. Figure 1A shows a light photomicrograph of a cross section of a WD HAE culture, illustrating the

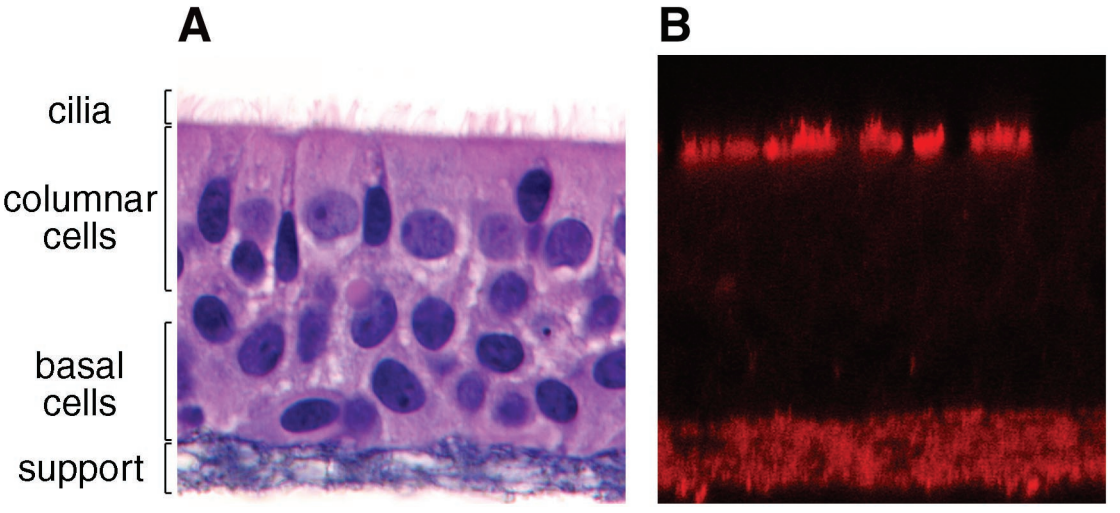

FIG. 1. Cell morphology and KS expression at the apical ciliated surfaces of WD HAE cell cultures. (A) Light micrograph of a cross section of a WD HAE culture grown at an ALI on a semipermeable membrane support for 4 weeks. Under these conditions, pseudostratified mucociliary epithelial cell morphology was generated. The cells were counterstained with hematoxylin and eosin. (B) Confocal fluorescent optical section of a live WD HAE culture exposed to an antibody specific for KS and detected with a secondary antibody conjugated to Texas Red. Note that KS serves as a marker for ciliated columnar epithelial cells at the apical surface of the culture and that the permeable support, a 10-μm-deep layer underlying the basal epithelial cells, displays non-KS-specific autofluorescence. Original magnification, ×100.

pseudostratified mucociliary epithelium with abundant ciliated cells. Approximately 25% of the total cells within a culture are lumenal cells. Confocal fluorescence optical sectioning of cultures probed with antibody to KS followed by a Texas Red-conjugated secondary antibody specifically identified the cilia of ciliated columnar airway epithelial cells (Fig. 1B).

Using this culture system, access to the apical and/or basolateral surface of the epithelium allowed investigation of whether rgRSV can infect the HAE via either surface. HAE cultures were inoculated with rgRSV (7×10^6 PFU; multiplicity of infection [MOI], ~20) applied to either the apical or basolateral surface for 1 h, washed, incubated for a further 24 h, and examined by fluorescence microscopy en face to detect expression of GFP as an indication of rgRSV infection. rgRSV infected HAE cells with high efficiency following inoculation of the apical surface, whereas inoculation of the basolateral surface resulted in little or no infection (Fig. 2). In contrast, as previously reported, AdVGFP (10^8 PFU; MOI, ~300) applied to the apical surface resulted in no GFP expression, whereas application to the basolateral surface resulted in efficient gene transfer.

Since RSV is pleomorphic and can vary in size, the possibility existed that the pore size of the Transwell-Col membrane support (0.4 μm) might restrict RSV access to the basolateral surfaces of the cultures. To investigate this possibility, we filtered rgRSV through 0.4-μm-pore-size Transwell-Col membrane supports positioned above the apical surfaces of WD HAE cultures. The efficiencies of rgRSV infection were similar whether rgRSV was applied directly to the apical surface or passed through the membrane support (data not shown), indicating that the inability of rgRSV to infect via the basolateral surface was not due to pore size restriction of the membrane support. These results show that rgRSV efficiently infects WD HAE cells via the apical but not the basolateral surface, which is the direct inverse of the polarized gene transfer characteristics of AdV.

For culture preparations from 10 different donors treated with the highest dose of rgRSV (7×10^6 PFU; MOI, ~20), a range of infection efficiencies was observed (30 to 80% of cells infected), with an average overall efficiency of ~52%. These data show that in this model of HAE, rgRSV is able to efficiently infect epithelial cells via the lumenal (apical) membrane, but they suggest that not all of the luminal cells were readily infected.

rgRSV specifically infects ciliated cells of the apical surface. In order to identify the cell types infected by rgRSV, HAE cultures were inoculated via the apical or basolateral surface with rgRSV as described above, incubated for 24 h, immunostained for KS, and visualized by confocal optical sectioning. Figure 3 shows that rgRSV-mediated GFP expression was localized to lumenal-surface columnar cells. Furthermore, the cells infected by rgRSV represented the ciliated subpopulation of lumenal cells. Although not every ciliated cell in a particular culture was infected by rgRSV, probably due to a limitation of rgRSV titer, those that were infected were exclusively ciliated cells. Basolateral inoculation of rgRSV resulted in little or no GFP expression in any cell type within the epithelium (<0.01% of cells). In contrast, parallel studies with AdVGFP revealed an absence of GFP expression following inoculation of the apical surface, whereas efficient expression was observed following basolateral inoculation, with basal cells as the preferential target cell type for AdV, as previously reported (31). These data suggest that, in an intact epithelium, rgRSV preferentially targets ciliated cells of the apical surface.

The results presented above indicated that the cell layer at the basal surface is refractory to rgRSV infection while the ciliated cells of the lumenal surface are readily infected. It was of interest to determine whether other cell types, e.g., inter-

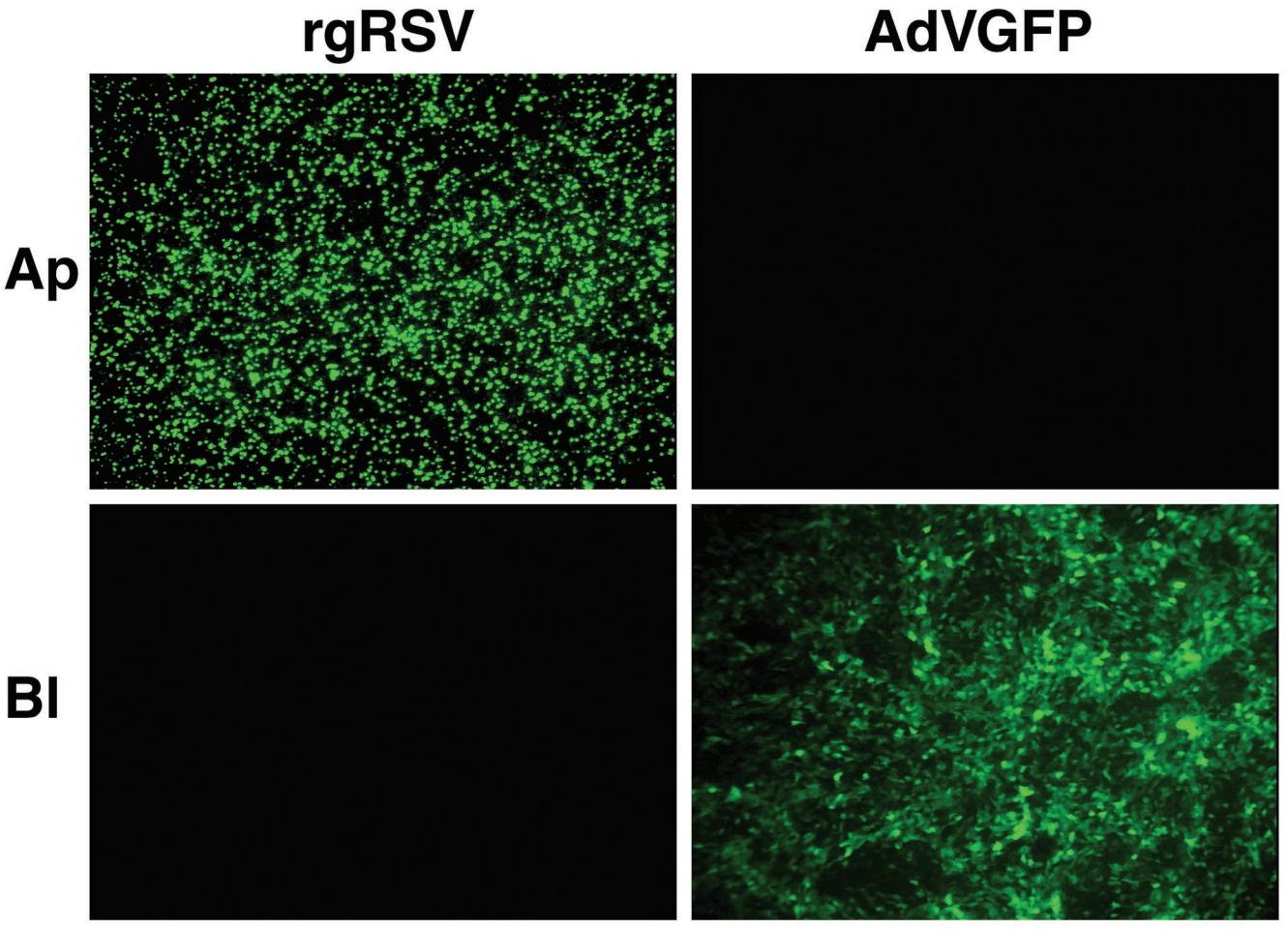

FIG. 2. Comparison of the abilities of rgRSV and AdVGFP to infect the apical (Ap) versus the basolateral (Bl) surfaces of WD HAE cultures. rgRSV (7×10^6 PFU; MOI, ~20) or AdVGFP (10^8 PFU; MOI, ~300) was applied to either the apical or basolateral surface of the cultures as detailed in Materials and Methods. Twenty-four hours later, the cultures were analyzed en face for GFP expression by fluorescence photomicroscopy. Original magnification, ×10.

mediate cells, within the multilayer WD HAE cultures could also be infected with rgRSV. This possibility was evaluated using an epithelium injury model that allows lumenally applied virus to reach underlying intermediate and basal epithelial cells. WD HAE cultures were mechanically injured with a pipette tip, followed immediately by inoculation with either rgRSV or AdVGFP on the apical surface for 1 h. The cultures were then incubated for 24 h, immunostained for KS, and visualized en face. As shown in Fig. 4, rgRSV infection occurred only in intact apical regions of the epithelium, coincident with KS staining, with few GFP-expressing cells present in the region of injury. In contrast, cultures inoculated with AdVGFP were transduced only within the region of injury, where basal cells were exposed. Thus, nonlumenal airway epithelial cells (basal and intermediate cells) exposed by mechanical damage were confirmed to lack KS, as expected, and were not susceptible to infection by rgRSV. In contrast, as described previously, HAE cells that underwent mechanical damage were readily infected by AdVGFP.

To further test the effect on rgRSV infection by disturbing the integrity of the lumenal cell layer, the epithelial cell tight junctions were transiently opened by the transient application of EGTA (10 mM) to the apical surface to allow virus access to the basolateral membranes of the cells. This treatment has been shown to produce a significant increase in AdV-mediated gene transfer, since opening the tight junctions allows access of AdV to basolaterally located receptors (7, 38). No differences were observed in the efficiency of rgRSV infection or in the cell type infected by rgRSV between cultures that maintained intact tight junctions and those in which tight junctions were transiently opened (data not shown). In sum, these results indicate that rgRSV specifically targets the apical surfaces of ciliated airway epithelial cells and that this tropism is not based on physical accessibility required for entry.

We also examined whether cultures generated from proximal and distal airway regions were also susceptible to rgRSV infection. For cultures prepared from nasal, tracheobronchial, and bronchiolar epithelium, rgRSV exhibited the pattern of infecting ciliated lumenal cells (data not shown). These results suggest that all regions of the conducting airway epithelium are susceptible to infection by rgRSV and that at each location rgRSV preferentially, and perhaps exclusively, infects ciliated cells.

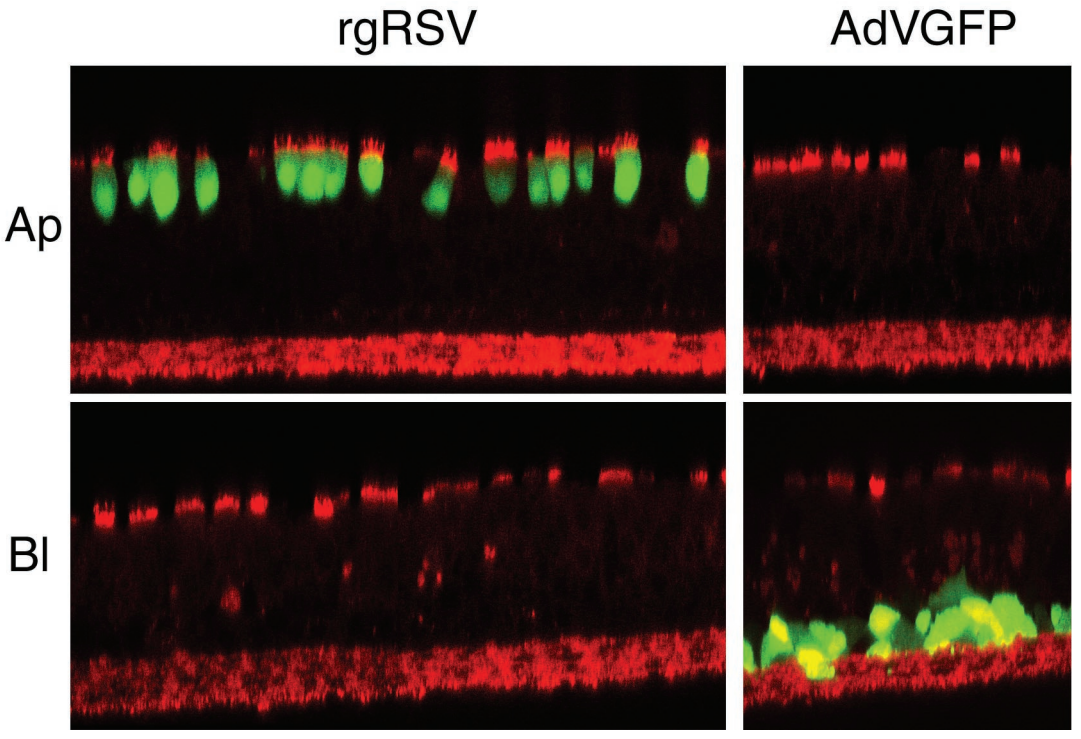

FIG. 3. Polarity of rgRSV infection of WD HAE cultures. Shown are confocal fluorescent-optical-section photomicrographs of HAE cultures inoculated via either the apical (Ap) or basolateral (Bl) surfaces with rgRSV or AdVGFP. Twenty-four hours after infection, the cultures were fixed and immunostained with antibody specific for KS and detected by a secondary antibody conjugated to Texas Red. The KS-expressing apical surfaces of ciliated cells are shown in red, and virus-infected cells are shown in green. Original magnification, ×63.

Susceptibility of HAE cultures to rgRSV infection requires differentiation and is coincident with ciliogenesis. We and others have previously shown that the apical surfaces of WD HAE cultures are resistant to AdV-mediated gene transfer because the receptors required for AdV entry are absent from the apical surfaces of airway epithelia (31, 44). However, poorly differentiated (PD) HAE cultures, which are confluent, immature cultures that are precursors to WD HAE, can be transduced by AdV with high efficiency due to the availability of AdV receptors and uptake mechanisms (31). To determine whether the HAE differentiation state affected susceptibility to rgRSV infection, we applied rgRSV to cultures at different stages in the differentiation process under otherwise identical conditions. PD HAE cultures that were inoculated with rgRSV had no evidence of GFP expression 24 to 48 h later (data not shown), indicating that these cells are not susceptible to rgRSV infection. In other cultures, after the establishment of an ALI, susceptibility to apical infection with rgRSV was evaluated as a function of time. As shown in Fig. 5A, very few cells were susceptible to infection on day 2, whereas susceptibility increased substantially by day 6, reaching a maximum on day 14. Interestingly, susceptibility to infection correlated with ciliogenesis of the columnar cells of the apical surface (Fig. 5B). These results indicate that rgRSV infection is differentiation dependent and that the extent of infection is directly related to the presence of ciliated columnar epithelial cells.

rgRSV infection, spread, and shedding occur at the apical surfaces of HAE cultures. Since rgRSV infects ciliated cells via the apical membrane, we investigated whether rgRSV was shed from the apical and/or basolateral surfaces of WD HAE cultures. The apical surfaces of cultures were inoculated with rgRSV, and infection was allowed to proceed over the following 7 days. On each day postinoculation, samples derived from either the apical or basolateral surfaces were obtained, and the amount of rgRSV in these samples was determined by standard titration on HEp-2 cells. For six individual cultures sampled for 7 days, in all cases rgRSV was shed only from the apical surfaces, as shown in Fig. 6. Within the limits of detection, no rgRSV was shed from the basolateral surfaces of HAE cultures. These data indicate that both the initial infection and subsequent virus shedding for rgRSV are polarized to the apical surfaces of ciliated cells in HAE.

To monitor the time course of rgRSV infection of WD HAE cells, cultures were inoculated at the apical surface with a small amount of virus (7×10^3 PFU), and fluorescence photomicrographs were taken en face at 1-day intervals to visualize GFP expression. At 24 h postinoculation, rgRSV infection resulted in a small number of individual green cells, as shown in Fig. 7A. The spread of rgRSV infection in the cultures over the next 24 h showed a vectorial pattern radiating from each focal infection point, forming a circular pattern of infection (Fig. 7B). This pattern was consistent with the pattern of ciliary movement in these cultures. Over the next 48 h, rgRSV replication and spread led to a large proportion (>80%) of infected cells (Fig. 7D).

These observations suggest that rgRSV buds from the apical surface and is released into the lumenal periciliary fluid and/or

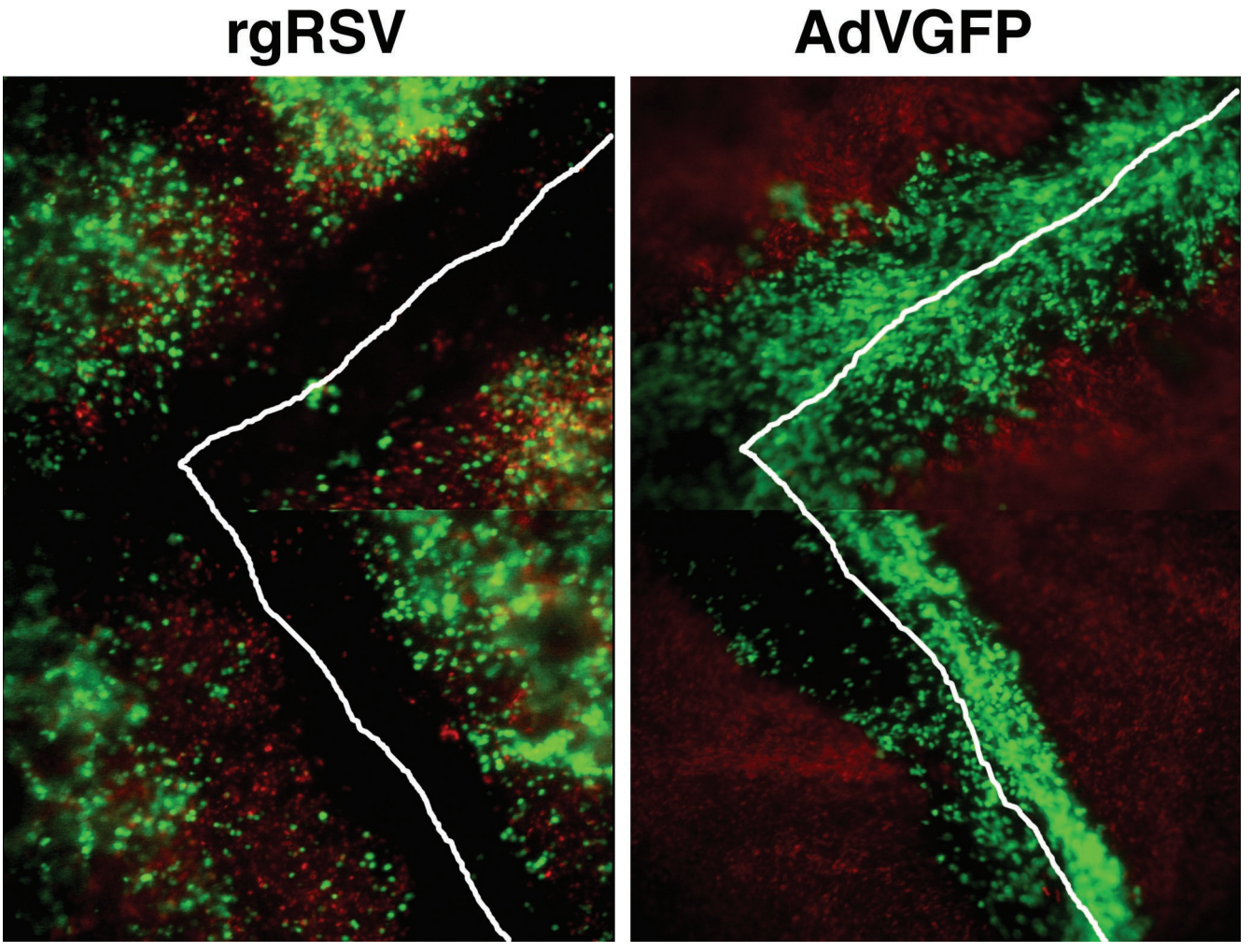

FIG. 4. Effect of mechanical damage to the epithelium of WD HAE cultures on rgRSV and AdVGFP infection. WD HAE cultures were scratched with a pipette tip across the apical surface to expose the underlying basal cells along the injury path (white lines). The apical surfaces of injured cultures were immediately inoculated with rgRSV or AdVGFP as for Fig. 2; 24 h later, the cultures were fixed and immunostained with antibody to KS, and the cultures were examined en face by fluorescence photomicroscopy. Note that in the left panel few cells expressed GFP within the injury path, while the undamaged regions colocalized with both GFP and KS expression, reflecting the presence of rgRSV infection and ciliated cells, respectively, in this region. In contrast, in the right panel the injury tract expressed GFP, reflecting AdVGFP infection, but did not colocalize with the undamaged tissue that expressed KS, reflecting the presence of intact ciliated cells. Original magnification, ×10.

overlying mucus layer. Thereafter, rgRSV is spread vectorially to adjacent cells within these compartments.

Effects of a neutralizing antibody and ribavirin on rgRSV infection. We further characterized the model by examining the effect of two clinically relevant antiviral agents effective against RSV infection. One agent, Synagis, is a humanized monoclonal antibody specific to the F protein that efficiently neutralizes viral infectivity. This antibody approach is currently in use in passive parenteral immunoprophylaxis in high-risk infants. The second agent, ribavirin, is a nucleoside analog that is used clinically as therapy for RSV infection. We tested the abilities of Synagis and ribavirin to inhibit both initial infection and subsequent spread of rgRSV in WD HAE cultures.

To determine whether these reagents could inhibit initial rgRSV infection, the antibody (250 μg/ml) was mixed with virus and applied to the apical surface, whereas ribavirin (100 μg/ml) was added to the basolateral medium immediately before inoculation. As shown in Fig. 8C and D, respectively, both the antibody and the ribavirin treatments resulted in complete inhibition of rgRSV infection compared to the control (Fig. 8A). To determine whether antibody and ribavirin were also able to reduce viral spread in cultures after infection with rgRSV, either antibody (apical) or ribavirin (basolateral) was applied 6 or 24 h, respectively, after inoculation of WD HAE with rgRSV. As expected, infected cells were detected 24 h postinoculation, but 3 days later there was no evidence of viral spread for cultures that received either the antibody (Fig. 8F) or ribavirin (Fig. 8H) treatment, in contrast to the rapid spread of rgRSV in the untreated cultures (Fig. 8B). Removal of antibody or ribavirin from the respective cultures allowed the resumption of rgRSV spread within 48 h (results not shown).

Additional experiments were performed to determine whether the anti-F antibody inhibited rgRSV infection and spread when the antibody was exposed to the basolateral

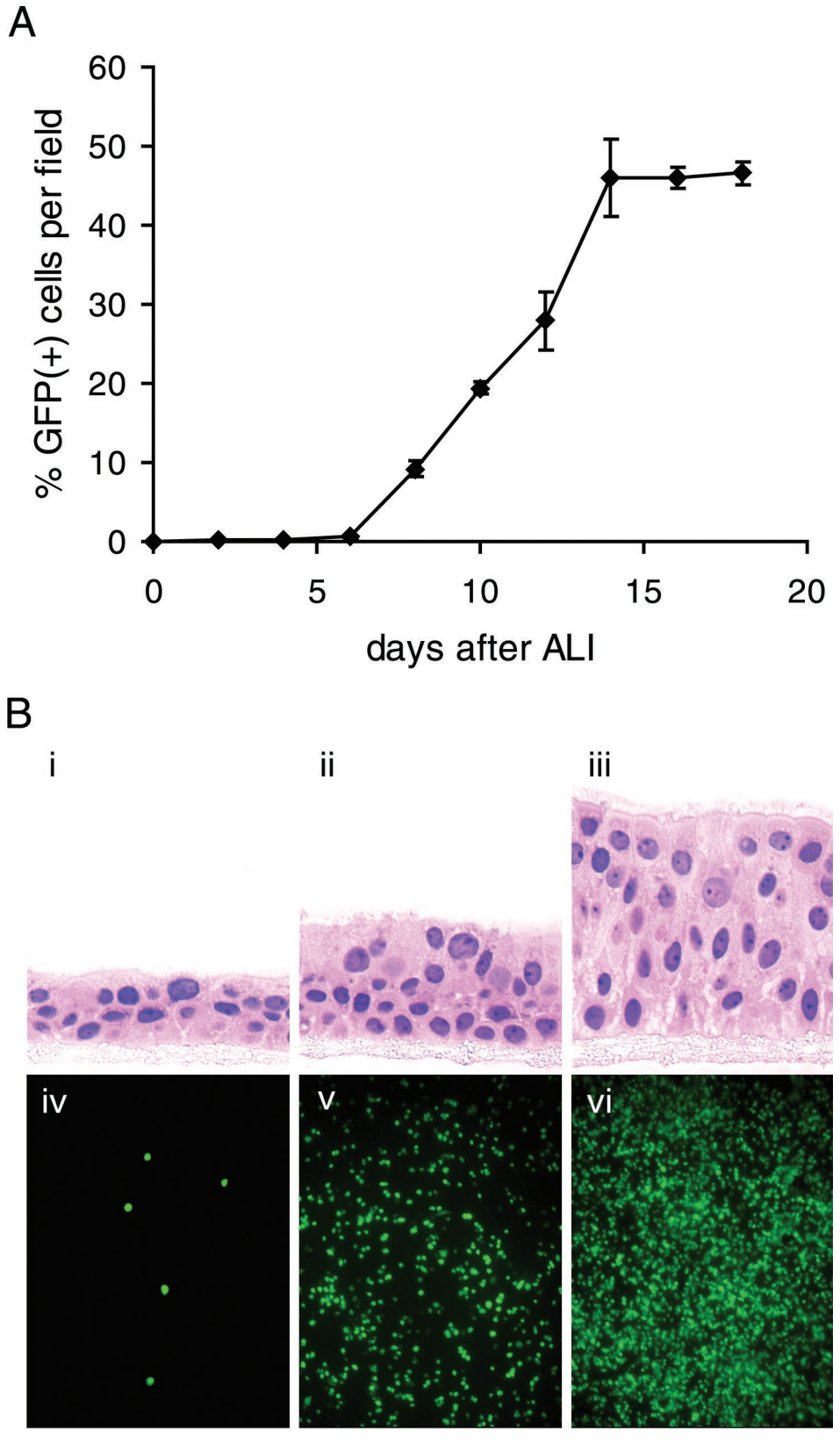

FIG. 5. Susceptibility of HAE cultures to rgRSV infection as a function of the differentiation state of the culture. (A) Freshly plated cells were grown to confluence to represent a PD cell type and allowed to differentiate with time. On the indicated days following establishment of an ALI, replicate cultures were inoculated with rgRSV (7×10^6 PFU), and the percentage of GFP-positive cells was quantitated by fluorescence photomicroscopy 24 h later. Each datum point represents the mean of three independent measurements ± standard error of the mean. (B) Representative photomicrographs of the differentiation status of HAE cultures on day 2 (i), day 8 (ii), and day 14 (iii) after initiation of an ALI. Note the abundant ciliated cells on day 14. The cells were counterstained with hematoxylin and eosin. Also shown are en face fluorescence photomicrographs of corresponding cultures expressing GFP 24 h after inoculation with rgRSV on day 2 (iv), day 8 (v), and day 14 (vi). Original magnifications, ×100 (light) and ×10 (fluorescence).

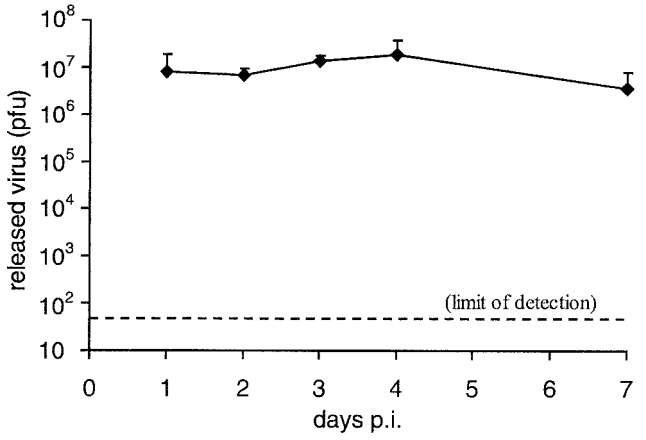

FIG. 6. Polarized release of rgRSV from the apical surfaces of WD HAE cultures. Virus shed from either the apical or basolateral surfaces of six independent cultures was collected at 24 h intervals as described in Materials and Methods. Titration of the collected samples on HEp-2 cells revealed significant shedding of rgRSV from the apical surface (diamonds), whereas within the limits of detection, no viral shedding was measured from the basolateral surface (below limits of detection). The values shown represent the mean ± standard deviation ($n = 6$).

rather than the apical surface. Antibody applied to the basolateral surface either at the time of or 24 h prior to rgRSV inoculation was not effective in reducing the infection or spread of rgRSV compared to control cultures (results not shown). These data illustrated the efficacy of these clinical strategies to reduce the infectivity of RSV in a model of HAE. In particular, these experiments illustrated that antibody applied to the luminal surface efficiently gained access to the local site of infection so that all viral spread was inhibited. These data are consistent with the efficacy of Synagis given parentally but point to the requirement for antibody to reach apical compartments in order to be effective therapeutically.

Persistence of rgRSV in HAE without obvious cytopathology. In general, the number of cells expressing GFP in WD HAE cultures peaked 2 to 3 days after initial infection, followed by a decrease in the number of positive cells over the next 36 days to a level approximately 25% of that at day 3, at which point the number of infected cells stabilized.

An example of GFP expression 36 days after rgRSV inoculation is shown in Fig. 9A. For periods of up to 3 months, the

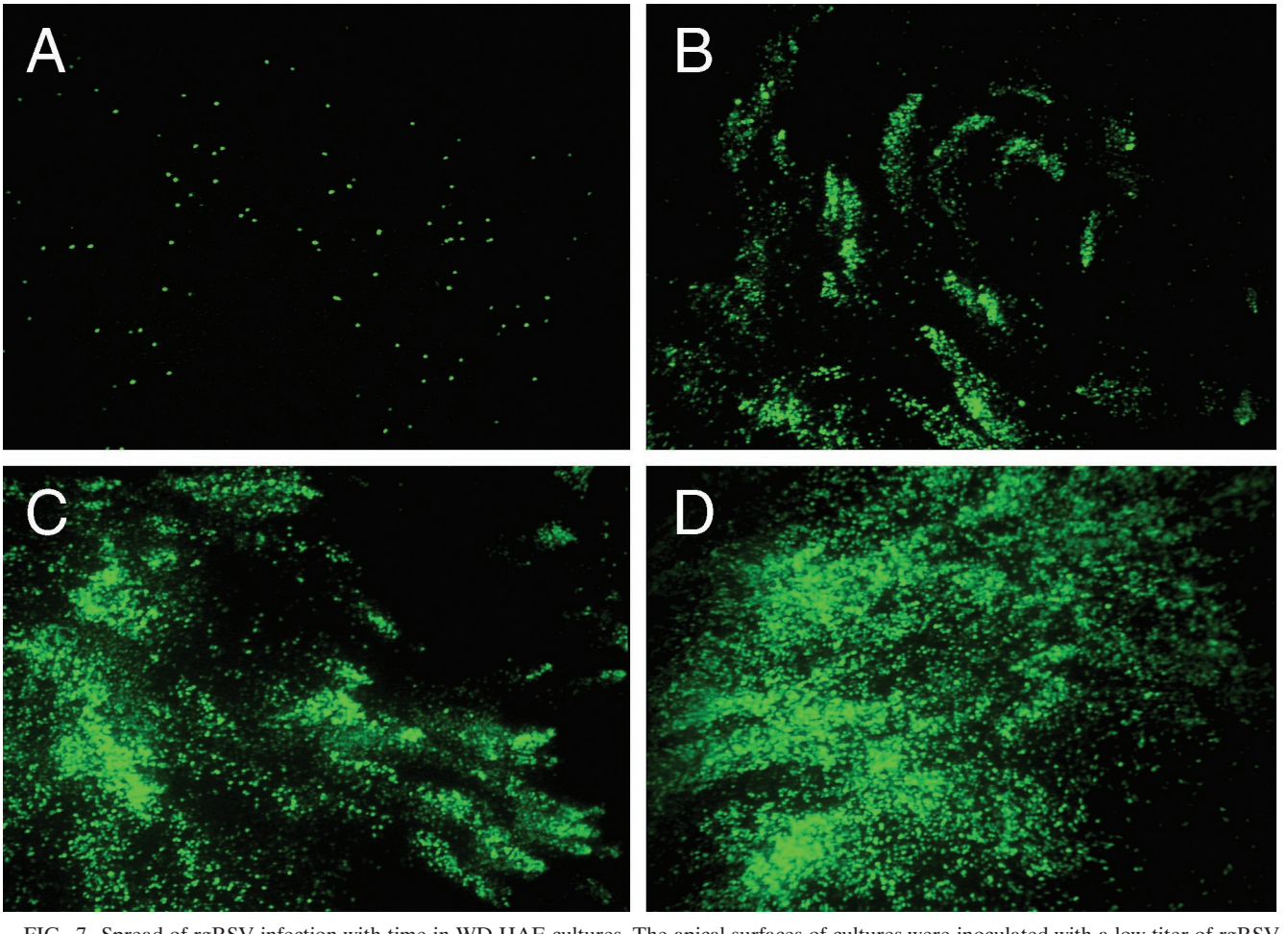

FIG. 7. Spread of rgRSV infection with time in WD HAE cultures. The apical surfaces of cultures were inoculated with a low titer of rgRSV (7×10^3 PFU) to achieve a submaximal number of cells expressing GFP at 24 h. Infection was then allowed to proceed over 4 days, and GFP expression was examined en face by fluorescence photomicroscopy on days 1 (A), 2 (B), 3 (C), and 4 (D) postinoculation. Note the counterclockwise circular spread of rgRSV infection by day 2 (B) and the increased number of rgRSV-infected cells by day 4. Original magnification, ×10.

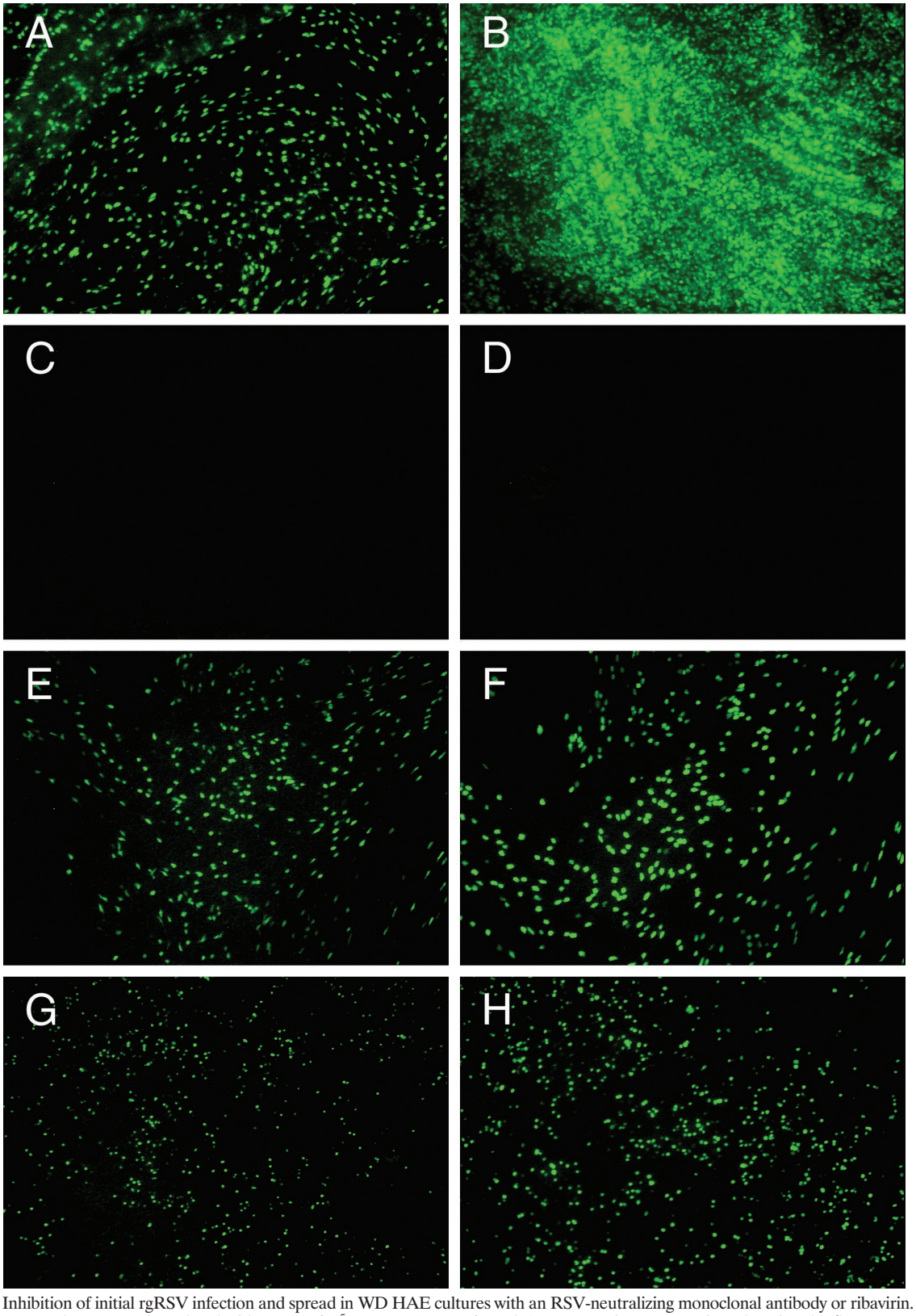

FIG. 8. Inhibition of initial rgRSV infection and spread in WD HAE cultures with an RSV-neutralizing monoclonal antibody or ribavirin. The apical surfaces of HAE cultures were inoculated with rgRSV (10^5 PFU; MOI, ~0.3), and GFP expression was monitored en face by fluorescence photomicroscopy 1 (A) and 3 (B) days later. To assess the effects of potential RSV inhibitors on initial rgRSV infection, parallel cultures were treated prior to rgRSV inoculation with either 250 μg of the F-specific RSV-neutralizing monoclonal antibody Synagis/ml applied to the apical surface (C) or 100 μg of ribavirin/ml included in the basolateral medium (D). The cultures were then inoculated with rgRSV as described above, and GFP expression was assessed 1 day later by fluorescence photomicroscopy. To assess the effects of RSV inhibitors on viral spread, parallel cultures were inoculated as described above and then treated with Synagis 6 h postinoculation, and GFP expression was assessed on day 1 (E) and day 3 (F) postinoculation. Cultures treated with ribavirin 24 h postinoculation were assessed for GFP expression by fluorescence photomicroscopy on day 2 (G) and day 4 (H) postinoculation. Original magnification, ×10.

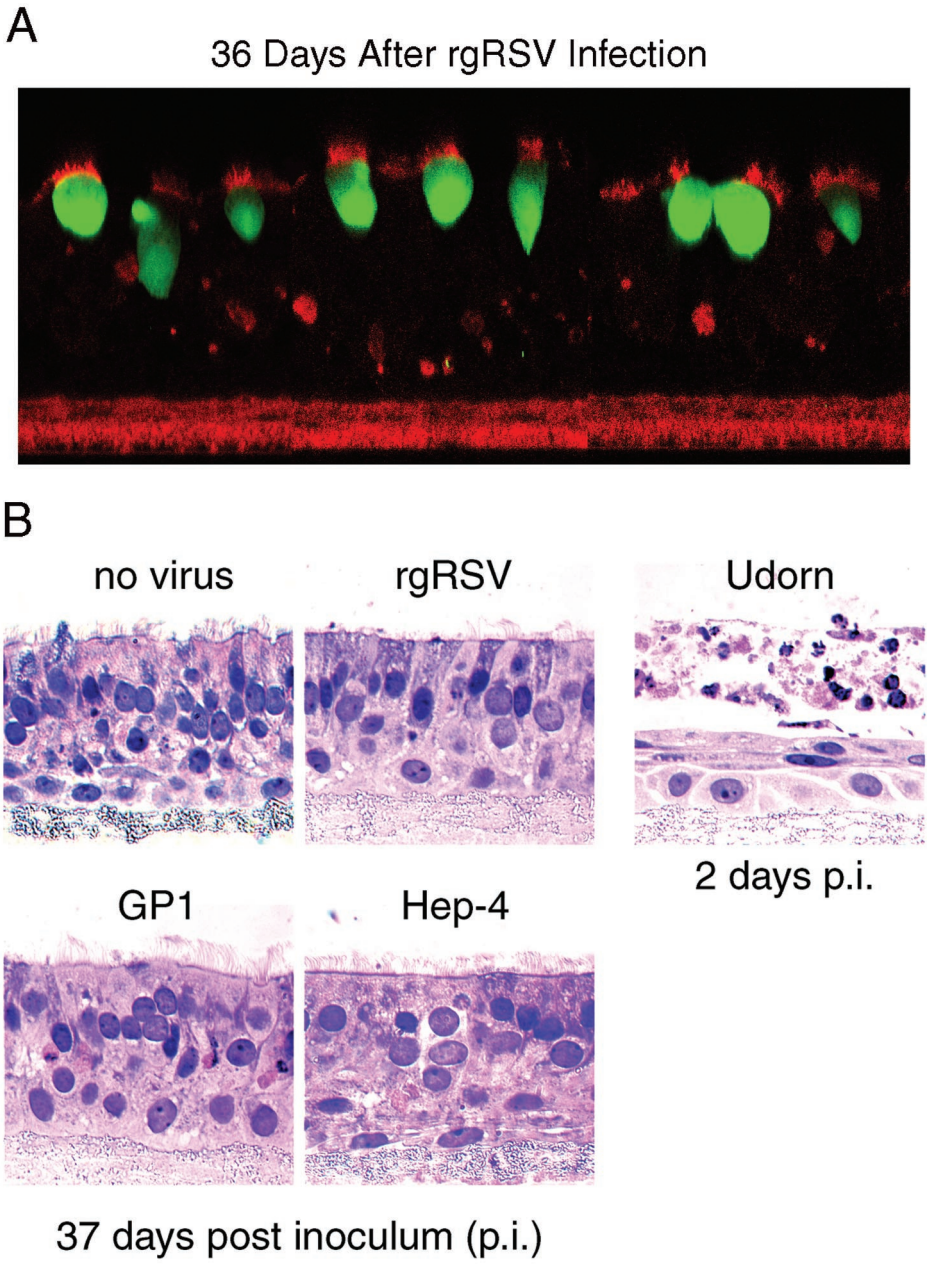

FIG. 9. Lack of RSV-specific obvious cytopathology in WD HAE cells. (A) Confocal optical section of rgRSV-mediated GFP expression 36 days after apical inoculation of a WD HAE culture with rgRSV (7×10^6 PFU). GFP expression (green) was predominately in ciliated cells, as shown by colocalization with KS-specific antibodies (apical red signal). Note the lack of cell-cell fusion, i.e., syncytium formation. Original magnification, ×63. (B) No obvious cytopathology of different RSV isolates was observed after apical inoculation of WD HAE cultures. The apical surfaces of HAE cultures were inoculated with either rgRSV (10^6 PFU); GP1, an isogenic recombinant RSV that lacks GFP (10^6 PFU); Hep-4, a biologically derived wild-type RSV (10^6 PFU); or the Udorn strain of influenza A virus (10^6 PFU). The RSV- and influenza virus-inoculated cultures were incubated for 37 and 2 days, respectively. Histological cross sections counterstained with hematoxylin and eosin showed no gross histological differences in cell morphology for the RSV-inoculated cultures compared to cultures not inoculated with any virus. In contrast, cultures inoculated with influenza A virus underwent significant cytopathology 2 days postinoculation. Original magnification, ×63.

longest interval studied, at the light microscope level, the histological integrity of rgRSV-infected cultures was not detectably altered compared to uninfected cultures from the same source. Specifically, cells appeared to be normal following rgRSV infection, there was no syncytium formation, and the cilial beat was visually unaltered. Histological examination of cultures infected by rgRSV for more than a month revealed no gross histological differences and, importantly, no cell fusion or syncytium formation (Fig. 9B). We also infected WD HAE with a wild-type recombinant RSV that lacks the GFP gene (GP1), the direct parent of rgRSV, and with biologically derived wild-type RSV (Hep-4). Over a period of 36 days, these

cultures also failed to display obvious cytopathology (Fig. 9B). In contrast, cultures infected with influenza A virus (Udorn strain) exhibited dramatic, rapid destruction and shedding of the columnar airway epithelial cells, as has been observed in vivo (14, 15, 41).

These data indicate that RSV, unlike influenza A virus, does not produce obvious cytopathology in airway epithelial cultures in the absence of a host immune response and suggest that in vivo, the pathogenic nature of RSV infection might in large part be mediated by the host immune response.

DISCUSSION

We have investigated RSV infection in a model of primary WD HAE cells by using a recombinant RSV that expresses GFP to monitor RSV infection in living cells. We found that rgRSV primarily, perhaps exclusively, targets the lumenal ciliated columnar airway epithelial cells via the apical surfaces of the cultures. In contrast, rgRSV infection was not observed after basolateral inoculation and was not enhanced by disruption of epithelial tight junctions or by mechanical damage to the tissue. These observations suggest that basal cells and the basolateral surfaces of columnar cells are not permissive for rgRSV infection. Progeny rgRSV were released from the apical surfaces of WD HAE cultures, and rgRSV infection spread via the lumenal surface to neighboring ciliated cells. A previous study showed that RSV was released from the apical membranes of polarized Vero cell monolayer cultures (34). However, since these cells are not derived from human tissue, are not ciliated, are immortalized, and form a single monolayer, it was important to confirm and extend these results in a primary, differentiated, multilayered, ciliated HAE culture. An interesting finding, only possible in a culture model such as that used in the present study, was that virus spread in HAE cells appeared to be affected by the directionality of the cilial beat. The ciliary beat has been shown to direct the movement of the periciliary fluid as well as the overlying mucus in this culture model (26).

A striking observation was that rgRSV appeared to primarily, and perhaps exclusively, infect ciliated cells. This was not only true in WD cultures but was also observed in immature HAE cultures undergoing differentiation, where susceptibility to rgRSV infection was dependent on differentiation and coincident with ciliogenesis. This may mean that the cilia themselves are directly necessary for rgRSV infection and might, for example, be the site of viral attachment. A direct role of cilia in RSV infection would be an important difference from infection of established cell lines, such as HEp-2 cells, which are highly permissive to RSV but are not ciliated. Even if cilia are not directly involved with RSV infection, the maturational requirement of primary HAE cells for susceptibility to rgRSV infection is interesting and contrasts with the ability of RSV to infect many established cell lines. One possibility is that infection of established cell lines proceeds by an alternative pathway, one that might not be representative of that which occurs for the airway epithelium in vivo. For example, it will be of interest to test whether the requirement for glycosaminoglycans for efficient RSV infection of established lines, such as HEp-2 cells, is also a requirement in the WD HAE cultures. WD HAE cultures likely will be the cell model of choice for examining the mechanisms of virus-host cell interactions important for initiating RSV infection, at least for the airway epithelium.

RSV infection initiates in the upper airway regions, after which the virus can spread to more distal airway regions and to the alveoli (11). Our data indicate that the characteristics of infection are similar for epithelial cell cultures derived from upper and lower airway epithelia, specifically the nasal, tracheobronchial, and bronchiolar regions. We did not test the ability of rgRSV to infect cells of the alveolar region, and thus, the observations that we describe apply strictly to the epithelium of the conducting airways. We found that for culture models from these airway regions, the extent of infection correlated with the number of ciliated cells present in the culture. Although ciliated cells of the upper airways differ in height and length of the cilial shaft from ciliated cells of the bronchioles, we found that both upper and lower ciliated cell types were susceptible to rgRSV infection.

Two clinical strategies currently in use against RSV infection, namely, an anti-F protein monoclonal antibody used in pediatric immunoprophylaxis and the nucleoside analog ribavirin used in RSV therapy (33), were efficacious against rgRSV infection in WD HAE cultures. Our experiments emphasized important aspects of the polarity of airway epithelium. The antibody was only effective at preventing rgRSV spread when presented on the apical surface. This finding suggests that trans-epithelial cell transport of this IgG antibody from the basolateral to the apical compartment was not efficient in vitro, at least not within the incubation period studied. It is also consistent with clinical experience: serum IgG antibodies are inefficiently transported to the lumenal surface of the respiratory tract, and RSV-neutralizing serum antibodies restrict RSV infection only when present at high titer (10, 32). In contrast, ribavirin was active with basolateral application mimicking parental administration in vivo. The ability of ribavirin to strongly inhibit RSV replication in vitro had been shown previously with nonpolarized cells (16). In the present work, the strong inhibition of GFP expression observed with ribavirin, added immediately before virus inoculation, shows that this nucleoside analog readily achieved bioavailability and greatly restricted gene expression. In addition, ribavirin acts early in the viral infection cycle, greatly restricting viral gene expression in addition to blocking the spread of infection.

An interesting finding of the present study was the observation that GFP expression was maintained in WD HAE cultures for up to 3 months, the longest period during which the cultures were maintained. It is not known whether the GFP-expressing cells observed after 3 months were ones that had been infected at the outset and maintained throughout the entire period or whether there was turnover of cells and reinfection of newly differentiated ciliated cells. Since the half-life of ciliated airway cells in these cultures has been estimated to be on the order of 40 to 100 days (1), it seems likely that some, and perhaps most, of the cells observed at 3 months were not among the infected cells present during inoculation. It is unknown whether rgRSV infection alters the half-life of ciliated airway cells in this culture system. However, it is clear that rgRSV infection produced little or no virus-specific obvious cytopathic effect in WD HAE cells. In particular, there was no evidence of syncytium formation in either columnar, intermediate, or basal cells. Thus, while rgRSV infection might have

subtle effects that were undetected, such as altering cellular half-life or inducing apoptosis, on a gross level rgRSV infection in this model of WD HAE was noncytopathic. We confirmed that this was not an artifact of rgRSV, since similar results were obtained with its wild-type recombinant parent lacking GFP as well as with a biologically derived wild-type RSV. The last two viruses have been shown to cause respiratory disease in chimpanzees, confirming that they are wild-type viruses (40).

In contrast to rgRSV, influenza A virus infection of WD HAE cultures resulted in rapid and extensive obvious cytopathology. This is consistent with clinical pathological findings that influenza virus infection results in extensive damage to the respiratory epithelium in infected humans (14, 15). These findings suggest that the tissue damage that is observed in the ciliated airway epithelium in vivo in response to RSV infection might not be due directly to viral presence and growth but rather is a consequence of destructive components of a robust immune response that eventually clears the virus infection. The situation was very different with influenza A virus, where virus infection was inherently highly cytopathic even in the absence of an immune response. Since we did not examine rgRSV infection in a system that corresponds to the alveolar epithelial region, it is possible that viral cytopathology might be different in that compartment.

The observed absence of obvious cytopathology by RSV infection in our culture model is in contrast to a previous report that also used a polarized, ciliated HAE cell culture model to assess the effect of wild-type RSV infection (37). In that study, RSV infection was found to have multiple effects: (i) lumenal cilial beat frequency was significantly inhibited within 2 h of virus addition to the lumenal surface, (ii) loss of cilia occurred 24 h after infection, (iii) syncytia formed, and (iv) all ciliated cells were sloughed from the culture by 3 to 5 days postinfection. Under similar conditions with our culture system, we did not observe any of these effects with rgRSV or with a wild-type RSV. The differences between these studies remain to be explained and may involve differences in the virus or cell culture systems used.

The lack of obvious RSV cytopathology with the HAE culture system described in this study is probably related to the lack of syncytium formation. Most studies of RSV have been performed with nonpolarized epithelial cell lines (e.g., HEp-2) that undergo cell-cell fusion 2 to 5 days after exposure to RSV and ultimately undergo cell death. However, using our polarized WD HAE model, no syncytia were ever observed. In vivo, syncytia have not been a prominent pathological feature of RSV infection, although they have been observed in autopsy tissue from RSV-infected patients (28), and RSV can cause giant-cell pneumonia in immunocompromised individuals (8). It might well be that, like our WD HAE cultures, the epithelium of the airways is refractory to syncytium formation and that which is observed in vivo involves the alveolar epithelium. In a study with the polarized epithelial cell line Vero C1008, Roberts et al. (34) reported that RSV matured at the apical surface without syncytium formation. They also found the F protein preferentially expressed at the apical surfaces of RSV-infected polarized cell monolayers, and it seems likely that the same is true of the multilayer HAE cultures in the present work. If the F protein is distributed exclusively at the apical surface, its interaction with adjacent cells likely would be restricted, and therefore the formation of syncytia would be suppressed. This segregation might not be an all-or-none phenomenon, as suggested by the data of Roberts et al. The efficiency of polar segregation might be further compromised under certain conditions and could be responsible for the syncytium formation observed by Tristram et al. (37). It also might be that the efficiency of polar segregation could be rendered less efficient in vivo, perhaps during an immune attack, which could account for the syncytia that are sometimes observed in vivo.

The ability of RSV to transfer genes to the ciliated cells of the airway epithelium after lumenal delivery indicates that this virus may provide a new vector system suitable for disorders of the lung epithelium, such as CF lung disease. In the case of CF, the ciliated airway epithelial cells are considered to be the target cell type that requires correction (2, 17, 20, 29). So far, gene therapy strategies have investigated vector systems such as adenovirus types 2 and 5, adeno-associated virus type 2, and retroviruses, none of which efficiently transfer genes to the lumenal columnar airway epithelial cells. The inefficiency of gene transfer is reported to be due to a number of barriers that are present at the apical surface of the lung epithelium, most notably the absence of receptors for the specific viruses from the apical surfaces of the cells (31, 44) and the presence of a restrictive glycocalyx barrier (30). For RSV, neither of these potential barriers appears to be restrictive for efficient infection.

Other vector systems have been reported to deliver genes to columnar airway cells via the lumenal membrane. Sendai virus vectors have been shown to infect ciliated cells of the rodent airway (43), but the efficiency with which this rodent virus infects HAE cells remains to be determined. Lentiviral vectors pseudotyped with components of Ebola virus membrane proteins have been shown to efficiently transduce HAE cells in vitro, suggesting that combining the efficiency of this system with the potential long duration of lentivirus-mediated gene expression may provide an effective vector for gene therapy strategies for the lung epithelium (19, 35). Interestingly, lentivirus pseudotyped with RSV F and G proteins did not transduce HAE cells, although there is some question as to the efficiency of virion formation with this pseudotyping strategy (19). The present study confirms that RSV indeed targets the lumenal epithelial cells. Coronavirus (39) and adeno-associated virus type 5 (45) have also been reported to be efficient for gene transfer to the lung epithelium. Further studies are required to determine the efficacies of these vectors for potential clinical applications.

The high efficiency of RSV infection in WD HAE cultures; the specificity of infection for the lumenal ciliated cells; the unexpected long duration of transgene expression, at least in the absence of an immune response; and the lack of obvious cytopathology in infected cultures indicate that RSV is an attractive candidate for further development as a gene transfer vector to target ciliated airway epithelium. Of course, further modification of the recombinant virus likely will be necessary for this application. For example, it will be necessary to use a version of recombinant RSV that does not cause respiratory tract disease. Fortunately, a wide array of recombinantly derived attenuated versions of RSV are available, and there is extensive clinical experience with experimental RSV vaccines that will aid in the development of vectors suitable for gene transfer to the lung epithelium.

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REFERENCES

1. **Blenkinsopp, W. K.** 1967. Proliferation of respiratory tract epithelium in the rat. Exp. Cell Res. **46:**144–154.
2. **Boucher, R. C.** 1999. Status of gene therapy for cystic fibrosis lung disease. J. Clin. Investig. **103:**441–445.
3. **Bukreyev, A., S. S. Whitehead, N. Bukreyeva, B. R. Murphy, and P. L. Collins.** 1999. Interferon gamma expressed by a recombinant respiratory syncytial virus attenuates virus replication in mice without compromising immunogenicity. Proc. Natl. Acad. Sci. USA **96:**2367–2372.
4. **Bukreyev, A., S. S. Whitehead, B. R. Murphy, and P. L. Collins.** 1997. Recombinant respiratory syncytial virus from which the entire SH gene has been deleted grows efficiently in cell culture and exhibits site-specific attenuation in the respiratory tract of the mouse. J. Virol. **71:**8973–8982.
5. **Collins, P. L., R. M. Chanock, and B. R. Murphy.** 2001. Respiratory syncytial virus, p. 1443–1485. *In* D. Knipe et al. (ed.), Virology, 4th ed. Raven Press, New York, N.Y.
6. **Collins, P. L., M. G. Hill, E. Camargo, H. Grosfeld, R. M. Chanock, and B. R. Murphy.** 1995. Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5′ proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. Proc. Natl. Acad. Sci. USA **92:**11563–11567.
7. **Coyne, C. B., M. M. Kelly, R. C. Boucher, and L. G. Johnson.** 2000. Enhanced epithelial gene transfer by modulation of tight junctions with sodium caprate. Am. J. Respir. Cell Mol. Biol. **23:**602–609.
8. **Delage, G., P. Brochu, L. Robillard, G. Jasmin, J. H. Joncas, and N. Lapointe.** 1984. Giant cell pneumonia due to respiratory syncytial virus. Occurrence in severe combined immunodeficiency syndrome. Arch. Pathol. Lab. Med. **108:**623–625.
9. **Feldman, S. A., S. Audet, and J. A. Beeler.** 2000. The fusion glycoprotein of human respiratory syncytial virus facilitates virus attachment and infectivity via an interaction with cellular heparan sulfate. J. Virol. **74:**6442–6447.
10. **Groothuis, J. R., M. J. Levin, W. Rodriguez, C. B. Hall, C. E. Long, H. W. Kim, B. A. Lauer, V. G. Hemming, et al.** 1991. Use of intravenous gamma globulin to passively immunize high-risk children against respiratory syncytial virus: safety and pharmacokinetics. Antimicrob. Agents Chemother. **35:**1469–1473.
11. **Hall, C. B.** 2001. Respiratory syncytial virus and parainfluenza virus. N. Engl. J. Med. **344:**1917–1928.
12. **Hallak, L. K., P. L. Collins, W. Knudson, and M. E. Peeples.** 2000. Iduronic acid-containing glycosaminoglycans on target cells are required for efficient respiratory syncytial virus infection. Virology **271:**264–275.
13. **Hallak, L. K., D. Spillmann, P. L. Collins, and M. E. Peeples.** 2000. Glycosaminoglycan sulfation requirements for respiratory syncytial virus infection. J. Virol. **74:**10508–10513.
14. **Hers, J. F.** 1966. Disturbances of the ciliated epithelium due to influenza virus. Am. Rev. Respir. Dis. **93**(Suppl.)**:**162–177.
15. **Hers, J. F., and J. Mulder.** 1961. Broad aspects of the pathology and pathogenesis of human influenza virus. Am. Rev. Respir. Dis. **83:**84–97.
16. **Hruska, J. F., J. M. Bernstein, R. G. Douglas, Jr., and C. B. Hall.** 1980. Effects of ribavirin on respiratory syncytial virus in vitro. Antimicrob. Agents Chemother. **17:**770–775.
17. **Kalin, N., A. Claass, M. Sommer, E. Puchelle, and B. Tummler.** 1999. ΔF508 CFTR protein expression in tissues from patients with cystic fibrosis. J. Clin. Investig. **103:**1379–1389.
18. **Karron, R. A., D. A. Buonagurio, A. F. Georgiu, S. S. Whitehead, J. E. Adamus, M. L. Clements-Mann, D. O. Harris, V. B. Randolph, S. A. Udem, B. R. Murphy, and M. S. Sidhu.** 1997. Respiratory syncytial virus (RSV) SH and G proteins are not essential for viral replication in vitro: clinical evaluation and molecular characterization of a cold-passaged, attenuated RSV subgroup B mutant. Proc. Natl. Acad. Sci. USA **94:**13961–13966.
19. **Kobinger, G. P., D. J. Weiner, Q. C. Yu, and J. M. Wilson.** 2001. Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. Nat. Biotechnol. **19:**225–230.
20. **Kreda, S. M., A. Mengos, T. Jensen, J. Riordan, and R. C. Boucher.** 2001. CFTR expression in normal and Δ-F508 CF airway epithelia. Pediatr. Pulmonol. Suppl. **22:**190.
21. **Krusat, T., and H. J. Streckert.** 1997. Heparin-dependent attachment of respiratory syncytial virus (RSV) to host cells. Arch. Virol. **142:**1247–1254.
22. **Levine, S., and R. Hamilton.** 1969. Kinetics of the respiratory syncytial virus growth cycle in HeLa cells. Arch. Gesamte Virusforsch. **28:**122–132.
23. **Levine, S., R. Klaiber-Franco, and P. R. Paradiso.** 1987. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. J. Gen. Virol. **68:**2521–2524.
24. **Martinez, I., and J. A. Melero.** 2000. Binding of human respiratory syncytial virus to cells: implication of sulfated cell surface proteoglycans. J. Gen. Virol. **81:**2715–2722.
25. **Matsui, H., B. R. Grubb, R. Tarran, S. H. Randell, J. T. Gatzy, C. W. Davis, and R. C. Boucher.** 1998. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. Cell **95:**1005–1015.
26. **Matsui, H., S. H. Randell, S. W. Peretti, C. W. Davis, and R. C. Boucher.** 1998. Coordinated clearance of periciliary liquid and mucus from airway surfaces. J. Clin. Investig. **102:**1125–1131.
27. **Murphy, B. R., A. V. Sotnikov, L. A. Lawrence, S. M. Banks, and G. A. Prince.** 1990. Enhanced pulmonary histopathology is observed in cotton rats immunized with formalin-inactivated respiratory syncytial virus (RSV) or purified F glycoprotein and challenged with RSV 3–6 months after immunization. Vaccine **8:**497–502.
28. **Neilson, K. A., and E. J. Yunis.** 1990. Demonstration of respiratory syncytial virus in an autopsy series. Pediatr. Pathol. **10:**491–502.
29. **Penque, D., F. Mendes, S. Beck, C. Farinha, P. Pacheco, P. Nogueira, J. Lavinha, R. Malho, and M. D. Amaral.** 2000. Cystic fibrosis F508del patients have apically localized CFTR in a reduced number of airway cells. Lab. Investig. **80:**857–868.
30. **Pickles, R. J., J. A. Fahrner, J. M. Petrella, R. C. Boucher, and J. M. Bergelson.** 2000. Retargeting the coxsackievirus and adenovirus receptor to the apical surface of polarized epithelial cells reveals the glycocalyx as a barrier to adenovirus-mediated gene transfer. J. Virol. **74:**6050–6057.
31. **Pickles, R. J., D. McCarty, H. Matsui, P. J. Hart, S. H. Randell, and R. C. Boucher.** 1998. Limited entry of adenovirus vectors into well-differentiated airway epithelium is responsible for inefficient gene transfer. J. Virol. **72:**6014–6023.
32. **Prince, G. A., V. G. Hemming, R. L. Horswood, P. A. Baron, and R. M. Chanock.** 1987. Effectiveness of topically administered neutralizing antibodies in experimental immunotherapy of respiratory syncytial virus infection in cotton rats. J. Virol. **61:**1851–1854.
33. **Rimar, J. M.** 1986. Ribavirin for treatment of RSV infection. MCN Am. J. Matern. Child Nurs. **11:**413.
34. **Roberts, S. R., R. W. Compans, and G. W. Wertz.** 1995. Respiratory syncytial virus matures at the apical surfaces of polarized epithelial cells. J. Virol. **69:**2667–2673.
35. **Sinn, P. L., M. A. Hickey, P. D. Staber, B. L. Davidson, D. A. Sanders, and P. B. McCray, Jr.** 2001. Pseudotyping feline immunodeficiency virus (FIV) with a filovirus glycoprotein improves apical gene transfer to differentiated human airway epithelia. Pediatr. Pulmonol. Suppl. **22:**244.
36. **Techaarpornkul, S., N. Barretto, and M. E. Peeples.** 2001. Functional analysis of recombinant respiratory syncytial virus deletion mutants lacking the small hydrophobic and/or attachment glycoprotein gene. J. Virol. **75:**6825–6834.
37. **Tristram, D. A., W. Hicks, Jr., and R. Hard.** 1998. Respiratory syncytial virus and human bronchial epithelium. Arch. Otolaryngol. Head Neck Surg. **124:**777–783.
38. **Walters, R. W., T. Grunst, J. M. Bergelson, R. W. Finberg, M. J. Welsh, and J. Zabner.** 1999. Basolateral localization of fiber receptors limits adenovirus infection from the apical surface of airway epithelia. J. Biol. Chem. **274:**10219–10226.
39. **Wang, G., C. Deering, M. Macke, J. Shao, R. Burns, D. M. Blau, K. V. Holmes, B. L. Davidson, S. Perlman, and P. B. McCray, Jr.** 2000. Human coronavirus 229E infects polarized airway epithelia from the apical surface. J. Virol. **74:**9234–9239.
40. **Whitehead, S. S., K. Juhasz, C. Y. Firestone, P. L. Collins, and B. R. Murphy.** 1998. Recombinant respiratory syncytial virus (RSV) bearing a set of mutations from cold-passaged RSV is attenuated in chimpanzees. J. Virol. **72:**4467–4471.
41. **Wright, P. F., and R. G. Webster.** 2001. Orthomyxoviruses, p. 1533–1579. *In* D. M. Knipe et al. (ed.), Fields virology. Lippincott Williams and Wilkins, Philadelphia, Pa.
42. **Wyatt, L. S., B. Moss, and S. Rozenblatt.** 1995. Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. Virology **210:**202–205.
43. **Yonemitsu, Y., C. Kitson, S. Ferrari, R. Farley, U. Griesenbach, D. Judd, R. Steel, P. Scheid, J. Zhu, P. K. Jeffery, A. Kato, M. K. Hasan, Y. Nagai, I. Masaki, M. Fukumura, M. Hasegawa, D. M. Geddes, and E. W. Alton.** 2000. Efficient gene transfer to airway epithelium using recombinant Sendai virus. Nat. Biotechnol. **18:**970–973.
44. **Zabner, J., P. Freimuth, A. Puga, A. Fabrega, and M. J. Welsh.** 1997. Lack of high affinity fiber receptor activity explains the resistance of ciliated airway epithelia to adenovirus infection. J. Clin. Investig. **100:**1144–1149.
45. **Zabner, J., M. Seiler, R. Walters, R. M. Kotin, W. Fulgeras, B. L. Davidson, and J. A. Chiorini.** 2000. Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. J. Virol. **74:**3852–3858.