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Inhibition of Human Metapneumovirus Binding to Heparan Sulfate Blocks Infection in Human Lung Cells and Airway Tissues

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ABSTRACT

Human metapneumovirus (HMPV), a recently discovered paramyxovirus, infects nearly 100% of the world population and causes severe respiratory disease in infants, the elderly, and immunocompromised patients. We previously showed that HMPV binds heparan sulfate proteoglycans (HSPGs) and that HMPV binding requires only the viral fusion (F) protein. To characterize the features of this interaction critical for HMPV binding and the role of this interaction in infection in relevant models, we utilized sulfated polysaccharides, heparan sulfate mimetics, and occluding compounds. Iota-carrageenan demonstrated potent anti-HMPV activity by inhibiting binding to lung cells mediated by the F protein. Furthermore, analysis of a minilibrary of variably sulfated derivatives of Escherichia coli K5 polysaccharide mimicking the HS structure revealed that the highly O-sulfated K5 polysaccharides inhibited HMPV infection, identifying a potential feature of HS critical for HMPV binding. The peptide dendrimer SB105-A10, which binds HS, reduced binding and infection in an F-dependent manner, suggesting that occlusion of HS at the target cell surface is sufficient to prevent infection. HMPV infection was also inhibited by these compounds during apical infection of polarized airway tissues, suggesting that these interactions take place during HMPV infection in a physiologically relevant model. These results reveal key features of the interaction between HMPV and HS, supporting the hypothesis that apical HS in the airway serves as a binding factor during infection, and HS modulating compounds may serve as a platform for potential antiviral development.

IMPORTANCE

Human metapneumovirus (HMPV) is a paramyxovirus that causes respiratory disease worldwide. It has been previously shown that HMPV requires binding to heparan sulfate on the surfaces of target cells for attachment and infection. In this study, we characterize the key features of this binding interaction using heparan sulfate mimetics, identify an important sulfate modification, and demonstrate that these interactions occur at the apical surface of polarized airway tissues. These findings provide insights into the initial binding step of HMPV infection that has potential for antiviral development.

Acute viral respiratory tract infection is the most frequently observed illness in humans worldwide (1). Human metapneumovirus (HMPV), an enveloped, negative-sense, single-stranded RNA virus in the Paramyxoviridae family, is a common cause of both upper and lower respiratory tract infections (2–4). First identified in 2001 in the Netherlands, HMPV is now known to be the cause of respiratory infections in humans since at least 1958 (2). Nearly every person is exposed to HMPV in the first decade of life; seroconversion occurs on average by the age of 5 years, and nearly 100% of individuals test seropositive for antibody reactivity to HMPV antigens by the age of 10 (5). In children, HMPV infection is the second most common cause of hospitalization due to respiratory infection after the closely related respiratory syncytial virus (RSV) (6, 7). Although infants are considered the most vulnerable population to illness from HMPV, adults can develop severe respiratory disease as well, especially the elderly, immunocompromised patients, and individuals with chronic underlying diseases (8–10). In addition to upper respiratory involvement with symptoms typically associated with the common cold, HMPV infection can result in serious lower respiratory syndromes such as pneumonia, bronchitis, and bronchiolitis (3, 11). Due to the recent ability to routinely detect this virus through the inclusion of HMPV in multiplex detection assays, HMPV has been associated with disease outside the respiratory tract in some cases, including viral encephalopathy (12–14) and acute myocarditis (15), from initial respiratory involvement. Despite this tremendous clinical burden, there is no known vaccine to prevent HMPV infection, and treatment options are limited to administering ribavirin, which does not have established efficacy against HMPV infection (16).

Key features of HMPV entry into target cells to establish infection have been characterized recently. HMPV utilizes heparan sulfate (HS) present on the cell surface to bind to target cells (17), followed by clathrin-mediated endocytosis and membrane fusion in endosomes (18). Integrin αVβ1 has also been shown to play a role for efficient HMPV entry (17, 19) and has been proposed to be involved in attachment (20). HS is a negatively charged polysaccharide belonging to the family of glycosaminoglycans composed of repeating disaccharide units formed by glucosamine and glucuronic acid, which can undergo a series of modifications dur-
ing the biosynthesis, leading to very heterogeneous chains. In HS, the glucosamine can be N-acetylated, or N-sulfated and O-sulfated, in various positions and to various degrees. Glucuronic acid can also be modified by epimerization.

HSPGs have been implicated in virus-cell interactions for other viruses, including RSV (21–23), human papillomavirus (HPV) (24), herpes simplex virus (HSV) (25–28), human immunodeficiency virus (HIV) (29–31), and others (reviewed in reference 32). We have previously shown that nearly complete reduction in HMPV binding and infection results when HS is removed from the cell surface using heparinases, whereas cells that are able to synthesize only HS, and not any other GAGs, are fully able to bind HMPV (17). Unlike other paramyxoviruses that require two distinct viral glycoproteins to mediate attachment and binding, the fusion protein (F) of HMPV is sufficient for binding and infection (33–35). Recombinant HMPV that does not have the attachment protein (G) or the small hydrophobic protein (SH) is able to bind cells at wild-type (WT) levels via HS (17). Thus, the putative interaction between the HMPV F and HS provides an opportunity for antiviral development.

In this study, we describe the potent anti-HMPV effects of the sulfated polysaccharide, iota-carrageenan, in models of respiratory epithelial cells and polarized airway tissues, indicating that the HS-F interaction is important in physiologically relevant models. To further characterize structural features of HS important for binding by HMPV F, we utilized a minilibrary of variably sulfated derivatives of Escherichia coli K5 polysaccharide, which revealed that the critical common feature required for effective inhibition of binding and infection is O-sulfation. In addition, we showed that occluding heparan sulfate with the peptide dendrimer SB105-A10 inhibits the binding interaction between HMPV F and target cells and airway tissues. These results provide additional support for a role for HS-HMPV F protein interactions in physiologically relevant models and identify key features of the interaction between HMPV and HS that have implications for infection in vivo and may serve for antiviral development.

MATERIALS AND METHODS

Cells and tissues. Vero cells were grown in Dulbecco modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (FBS). A549 cells were grown in Roswell Park Memorial Institute medium (RPMI; Lonza) supplemented with 10% FBS. BEAS-2B cells, a human bronchial epithelial cell line, obtained from ATCC, were maintained in BEGM medium containing all the recommended supplements (Lonza) in flasks coated with bovine collagen (Sigma), bovine fibronectin (VWR Scientific), and bovine serum albumin (EMD Millipore). All cells were grown at 37°C under 5% CO2.

Well-differentiated (transepithelial resistance > 1000 Ω) primary normal bronchial human airway epithelial (HAE) tissue cultures were purchased from MatTek Corp. (Ashland, MA). Cell culture inserts were placed atop two washers (MatTek) in six-well plates with 5 ml of AIR 100 growth medium (MatTek) in contact with the basal surface and incubated at an air-liquid interface at 37°C and 5% CO2. Tissues were maintained for 5 to 7 days for differentiation after arrival by washing the apical surface with 0.9% sodium chloride and changing the media every 48 h prior to initiation of the infection experiments.

Antibodies. A rabbit polyclonal antibody against avian metapneumovirus (AMPV) C matrix (M) protein supplied by Sagay Goyal (University of Minnesota) with cross-reactivity to HMPV M was used to detect HMPV M protein by Western blotting (36). Antipeptide antibodies to HMPV F (Genemed Synthesis, San Francisco, CA) were generated using amino acids 524 to 538 of HMPV F (37). All other antibodies were purchased from the various companies: β-actin (Sigma) and peroxidase AfiniPure goat anti-rabbit IgG and goat anti-mouse IgG (Jackson ImmunoResearch).

Heparan sulfate mimicking and occluding compounds. Iota-carrageenan was purchased from Sigma (Invitrogen). Peptide dendrimer SB105-A10 ([H-ASLVRK], Lys2-Lys-β-Ala-Oh) was synthesized by Lifetein with a purity of >95%. Escherichia coli K5 polysaccharides derivatives were provided by David Lembo and Glycores 2000 (38).

Cell viability assay. Approximately 10,000 BEAS-2B or A549 cells were grown in triplicate overnight in a 96-well plate. BEAS-2B cells were either incubated with 2 μM SB105-A10 for 1 h, 40 μg of iota-carrageenan/ml for 4 h, or 10 μM concentrations of each of the K5 derivatives for 4 h at 37°C. A549 cells were incubated with 2 μM SB105-A10 for 1 h at 37°C. Control cells were incubated with Opti-MEM, which was used to dilute all of the compounds. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Fisher Scientific) (5 mg/ml) was added, followed by incubation for 3 h at 37°C. The medium was removed from the cells by tapping the plate and blotting the excess liquid. Next, 100 μl of stop solution (90% isopropyl alcohol, 10% dimethyl sulfoxide) was added, and the plate was incubated at room temperature for 20 min in the dark with rocking. The absorbance was read at 590 nm using a plate reader. The absorbance of treated cells was normalized to the untreated control.

Virus propagation and titers. The recombinant, green fluorescent protein (GFP)-expressing HMPV (rgHMPV) strain CAN97-83 (genotype group A2) and the mutant viruses HMPV ΔG and HMPV ΔG/ΔSH with a codon-stabilized SH gene (39) were kindly provided by Peter L. Collins and Ursula J. Buchholz (NIADD, Bethesda, MD). The viruses were propagated in Vero cells (starting multiplicity of infection [MOI], 0.01 to 0.03), followed by incubation at 37°C with Opti-MEM, 200 mM 1-glutamine, and 0.3 μg of TPCK (tolyisulfonil phenylalanine chloromethyl ketone)-trypsin (Sigma)/ml, replenished every day. On day 5, or when cytopathic effects were observed in at least 25% of the cells, the cells and medium were collected and subjected to centrifugation at 2,500 × g for 10 min at 4°C on a Sorvall R17 tabletop centrifuge. The supernatant was then stored in 1× sucrose phosphate glutamate (SPG; 218 mM sucrose, 0.0049 M 1-glutamic acid, 0.0038 M KH2PO4, 0.0072 M K2HPO4), and aliquots were flash frozen in liquid nitrogen and thawed twice prior to storage at −80°C. To achieve more concentrated rgHMPV for HAE tissue experiments, supernatants of harvested cells and medium were subjected to centrifugation at 2,500 × g for 10 min at 4°C on a Sorvall R17 tabletop centrifuge. The supernatant was then stored in 1× sucrose phosphate glutamate (SPG; 218 mM sucrose, 0.0049 M 1-glutamic acid, 0.0038 M KH2PO4, 0.0072 M K2HPO4), and aliquots were flash frozen in liquid nitrogen and thawed twice prior to storage at −80°C. To achieve more concentrated rgHMPV for HAE tissue experiments, supernatants of harvested cells and medium were subjected to centrifugation at 20% sucrose cushion for 3 h at 27,000 × g at 4°C using a SW28 swinging-bucket rotor on a Beckman Optima L90-K ultracentrifuge. After centrifugation, the supernatant was removed, and the pellet was resuspended in 100 μl of Opti-MEM per T25 flask, harvested, and left at 4°C overnight. Aliquots were stored at −80°C by flash freezing in liquid nitrogen.

Recombinant GFP-expressing parainfluenza virus 5 (rgPIV5) was kindly provided by Robert Lamb (Howard Hughes Medical Institute, Northwestern University) (35). rgPIV5 was propagated in MDBK cells as described previously (40) and stored in 1× SPG. Aliquots were frozen in liquid nitrogen and thawed twice prior to storage at −80°C.

For GFP-expressing viruses (rgHMPV and rgPIV5), viral titers were calculated by creating serial dilutions of the viral samples in Opti-MEM. Vero cells were seeded on a 96-well plate overnight and infected in serial dilution (10−1 to 10−12) with 50 μl of virus solution in duplicate. The number of GFP-expressing cells was counted in wells demonstrating 25 to 100 GFP-positive cells the following day. The average titer was calculated based on the dilution of the virus solution in the wells counted.

For non-GFP expressing viruses (HMPV ΔG and HMPV ΔG/ΔSH) viral particles were estimated using serial dilutions of viral samples assessed for M protein content using Western blotting and compared to protein levels of an rgHMPV standard of known titer. The volumes of uninfected virus and the rgHMPV standard of a known concentration (2, 4, and 8 μl in duplicate) were resolved using SDS–15% PAGE, and the proteins were transferred to a polyvinylidene difluoride membrane (Fisher).
Blocking HS Binding Inhibits HMPV Infection

RESULTS

Iota-carrageenan inhibits HMPV infection in human respiratory cells. To dissect how interaction with HS regulates HMPV infection in physiologically relevant models and to determine whether blocking this interaction could be a potential antiviral approach, we utilized a sulfated polysaccharide, heparan sulfate mimics and a compound that occludes HS in combination with infection studies in human bronchial epithelial cells (BEAS-2B) or human airway epithelial (HAE) models. Sulfated polysaccharides have been previously used to target viral infection, including a number of studies with carrageenans, which are isolated from red seaweed (42). Carrageenans are composed of sulfated repeating galactose units (Fig. 1B). The three types of known carrageenans ( iota, lambda, and kappa) differ in number and positions of sulfate groups (reviewed in reference 42). Carrageenans have shown antiviral activity against a number of viral pathogens, including HPV (43), HIV (44), dengue virus (45), and influenza A virus (46). Importantly, iota-carrageenan has been used safely in human trials in the form of a nasal spray to reduce viral infection (47–49).

To verify that sulfated polysaccharides would inhibit HMPV infection in a relevant cell culture model, we first determined the anti-HMPV activity of iota-carrageenan, a well-characterized sulfated polysaccharide that has been shown to inhibit the infection of other viruses that bind HS. Infection of BEAS-2B cells was performed using a recombinant HMPV (strain CAN97-83, clade A2) that results in green fluorescent protein (GFP) expression as a surrogate measure for infectivity, which was quantified by flow cytometry. Pretreatment of HMPV with iota-carrageenan resulted in inhibition of infection, with nearly a complete reduction in infection achieved with 10 μg of iota-carrageenan/ml (Fig. 2A and C). To determine whether iota-carrageenan had a nonspecific antiviral effect, paramyxovirus parainfluenza virus 5 (PIV5), which does not utilize HS for binding, was used. Incubating rgPIV5, a recombinant PIV5 virus that results in GFP expression, with iota-carrageenan prior to infection did not inhibit infection of BEAS-2B cells at any of the concentrations used compared to the untreated control (Fig. 2B and C). Similar effects of iota-carrageenan on rgHMPV and rgPIV5 infection were observed in Vero cells (data not shown). These results indicate that HS-HMPV interactions are critical in BEAS-2B cells and that iota-
carrageenan has anti-HMPV activity, most likely attributed to its sulfated structure.

Our previous studies suggested that HMPV binds to HS via the F protein, and we hypothesized that iota-carrageenan is inhibiting HMPV infection by competing with HS binding sites located in HMPV F. To test this, HMPV was added to BEAS-2B cells at 4°C, allowing for binding to occur but not infection, and the amount of bound virus was quantified by detection of the HMPV matrix protein present in cell lysates by Western blotting, with β-actin as a loading control. Pretreating HMPV at an MOI of 1 with 40 μg of carrageenan/ml for 30 min at 4°C prior to addition to the cells resulted in about 85% inhibition of particle binding compared to the untreated control (Fig. 2D and E), demonstrating that iota-carrageenan competes with the binding of HMPV to HS. Of the three glycoproteins in the viral envelope of HMPV (the attachment protein G, the small hydrophobic protein SH, and F), only F is required by HMPV to be infectious; recombinant HMPVs without G or SH, HMPV ΔG and ΔGSH, respectively, are able to bind cells at wild-type levels (17) and are replication competent in a nonhuman primate model of infection (34). To test whether iota-carrageenan inhibition of HMPV binding and infection is dependent exclusively on F, we pretreated the recombinant HMPV ΔG and ΔGSH with iota-carrageenan and determined the effects on overall infection and binding in BEAS-2B cells. Iota-carrageenan inhibited HMPV ΔG and ΔGSH binding to a degree similar to that demonstrated by wild-type HMPV (Fig. 2D and E), which supports the hypothesis that interaction between HMPV and HS is mediated by F. Interestingly, a greater fraction of input particles of ΔG and ΔGSH HMPV than WT HMPV bound to BEAS-2B cells, suggesting that ΔG and ΔGSH HMPV binding to BEAS-2B cells was more efficient on a per particle basis (Fig. 3A). The input number of particles for all three virus samples was verified for each experiment and was not statistically different across all binding assays completed (Fig. 3B), demonstrating that increases in particle numbers did not cause the observed increase in binding for ΔG and ΔGSH HMPV. To address the possibility that the ΔG and ΔGSH recombinant viruses had greater F content that contributes to increased binding in BEAS-2B cells, the ratio of F to M protein in WT, ΔG, and ΔGSH HMPVs was determined by Western blotting (Fig. 3C). No difference in the F/M ratio was detected between WT HMPV and the recombinant viruses, indicating that increased binding is not due to increased per particle F protein levels (Fig. 3D). Thus, the reasons for these unexpected results remain to be determined. However, despite increased binding at the baseline, iota-carrageenan did inhibit the binding of ΔG and ΔGSH HMPVs to the same degree as did WT HMPV (Fig. 2E). These findings suggest that iota-carrageenan inhibits the binding of WT and recombinant HMPVs without G and SH in a manner that is dependent on F and not the other surface glycoproteins.

The results in a monolayer respiratory cell model support the hypothesis that HMPV F mediates a key binding interaction to HS, and this event can be inhibited using the highly sulfated polysaccharide, iota-carrageenan. However, a monolayer cell culture model is limited in the ability to recapitulate the complex features of the respiratory epithelium, which include moving cilia, mucus, distinct cell types with important physiological roles, and polarity maintained by tight junctions. Furthermore, immortalized cells highly express HSPGs in a pattern that may be different than complex organized tissues found in vivo, and immunohistochemistry studies have not detected significant amounts of HS on the apical surface of human airway, raising concerns that HS interactions may be less important in an airway model (50). We therefore examined the effect of sulfated polysaccharides in a polarized human airway tissue model (HAE; MatTek) that more closely recapitulates the complexity of the human airway, which is the primary site of HMPV infection. HAE tissues have been previously used as models of respiratory virus infection, including human
FIG 2  Iota-carrageenan inhibits HMPV infection in cells and tissues by blocking binding. (A) BEAS-2B cells infected with rgHMPV at an MOI of 1 was treated with variable concentrations of iota-carrageenan. Cells were imaged 24 HPI. (B) BEAS-2B cells were infected with rgPIV5 at an MOI of 1 treated with 40 μg of iota-carrageenan/ml or vehicle. Cells were imaged at 24 h postinfection. (C) Quantification of rgHMPV and rgPIV5 infection in BEAS-2B cells using flow cytometry to detect GFP expressing cells 24 h postinfection. The data are presented as a percent infection of the untreated control (0 μg/ml) for each virus. Data points are means ± the standard deviations of duplicate measurements and are representative of a minimum of three independent experiments. *, Statistical significance (P < 0.0001). (D) HMPV viruses (WT and recombinant mutants ΔG and ΔGΔSH) were treated with vehicle or 40 μg of iota-carrageenan/ml and added to BEAS-2B cells at an MOI of 1 at 4°C for particle binding. Lysates of washed cells were analyzed for HMPV binding by Western blotting for M. Input represents 5% WT HMPV was added to the cells for binding. No virus was added to mock-infected cells. β-Actin served as a loading control. (E) Band intensities of the matrix protein and β-actin were determined for untreated and treated (40 μg/ml iota-carrageenan) samples. The data are reported as a ratio of M to β-actin normalized to the untreated control for each virus. Data points are means ± the standard deviations of measurements representative of seven independent experiments. *, Statistical significance (P < 0.0001). (F) HAE tissues were infected with rgHMPV or rgPIV5 at an MOI of 5 treated with 40 μg of iota-carrageenan/ml or vehicle and imaged at 48 h postinfection at ×5 magnification. (G) Quantification of HAE tissue infection. Data points are means ± the standard deviations of triplicate measurements and are representative of a minimum three independent HMPV infection experiments. *, Statistical significance (P < 0.0001).
parainfluenza virus 3 (51), rhinovirus (52), human bocavirus 1 (53), and RSV (54).

To test whether iota-carrageenan inhibits HMPV infection in the HAE model, iota-carrageenan-treated rgHMPV was used to inoculate the apical surfaces of the HAE tissues, and a significant reduction in infection of approximately 75% was observed (Fig. 2F and G). Unlike rgHMPV, treatment of rgPIV5 with iota-carrageenan had no effect on infection in the airway tissues (Fig. 2F and G). These findings indicate that HMPV interactions with HS are also important in complex polarized airway tissues that histologically resemble the human respiratory tract and support the hypothesis that HMPV requires HS to establish infection at the apical surface of the respiratory epithelium.

O-sulfated K5 polysaccharide derivatives inhibit HMPV infection. Although our preliminary results strongly support a key role for HS in HMPV infection, the specific features of HS required remain to be determined. A class of molecules mimicking HS and thus possible inhibitors of HS-virus interactions is represented by the sulfated derivatives of the *E. coli* capsular K5 polysaccharide (Fig. 1D). K5 polysaccharide derivatives are heparan-like molecules devoid of anticoagulant activity obtained by the sulfation of the *E. coli* capsular K5 polysaccharide that has the same structure of the biosynthetic precursor of HS, N-acetyl heparosan. A small library of derivatives with different degrees of sulfation has been synthesized using chemical and enzymatic modifications (38). Sulfated K5 derivatives have been shown to inhibit infection in other viruses in a specific manner, including HPV (43), RSV (55), dengue virus (56), cytomegalovirus (CMV) (57), HSV-1 and HSV-2 (58), and HIV (59). Analysis of the anti-HMPV activity of these compounds can therefore be used to identify structural features that are important for recognition by HMPV F and potentially help to identify a potent HS mimic.

Because HS is negatively charged due to sulfate modifications on the disaccharide units, we hypothesized that charge-charge interactions contribute to the binding between F and this polysaccharide. Therefore, we predicted that the most highly sulfated K5 derivatives, mainly K5-NS(H) and K5-OS(H), would have the greatest inhibitory effect on HMPV infection. To test this, rgHMPV at an MOI of 1 pretreated with the derivatives at 1 μM was used to inoculate BEAS-2B cells. As predicted, the highly sulfated K5 derivatives, K5-NS(H) and K5-OS(H), dramatically inhibited infection (Fig. 4A). Among the lower sulfated derivatives, K5 and K5-NS did not have an observable effect on HMPV infection when examined by microscopy (Fig. 4A), whereas K5-N,OS(L) and K5-OS(L) (Fig. 1D) also inhibited HMPV infection dramatically. K5-NS, which has a single sulfate in position 2 of glucosamine, had no effect on HMPV infection, indicating a key role of O-sulfate groups in the observed inhibition. When HMPV was treated with variable concentrations (10 nm to 1 μM) of the K5 derivatives and used to infect BEAS-2B cells, flow cytometry analysis of infected cells revealed a dose-dependent inhibition of HMPV infection by all the O-sulfated K5 derivatives (Fig. 4B). K5-NS had no effect on HMPV infection, whereas some inhibition resulted from K5, although only at the highest concen-

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**FIG 3** Recombinant ΔG and ΔGΔSH HMPV exhibit enhanced binding to BEAS-2B cells compared to WT HMPV. (A) HMPV viruses (WT and recombinant mutants ΔG and ΔGΔSH) were added to BEAS-2B cells at an MOI of 1 at 4°C for particle binding. Lysates of washed cells and 5% of each virus volume added to cells for binding were analyzed for HMPV binding by Western blotting for M. The band intensities of the matrix protein and β-actin were determined for input and bound. The data are reported as a ratio of the percent bound M to the input. Data points are means ± the standard deviations of measurements representative of five independent experiments. *, Statistical significance (P < 0.05). (B) The input of the three viruses was determined by Western blotting by detection of the binding protein. The data are reported as an estimated number of particles added to cells in the binding assays. Data points are means ± the standard deviations of measurements representative of three different experiments. (C) HMPV viruses (WT and recombinant mutants ΔG and ΔGΔSH) were analyzed by Western blotting for F and M. (D) The relative band intensities were quantified. The data are reported as a ratio of the mean F to the mean M abundance and are representative of three independent experiments.
FIG 4 O-sulfated K5 polysaccharide derivatives inhibit HMPV infection in BEAS-2B cells by competing for binding. (A) BEAS-2B cells were infected with
rgHMPV at an MOI of 1 treated with a 1 μM concentration of each K5 polysaccharide derivative or vehicle. The cells were imaged at 24 h postinfection. BEAS-2B
cells were infected with rgHMPV (B) or rgPIV5 (C) at an MOI of 1 treated with a 10 nM, 100 nM, or 1 μM concentration of each K5 polysaccharide derivative
or vehicle. Infection was quantified by flow cytometry to detect GFP-expressing cells at 24 h postinfection. The data are presented as the percent infection of the
untreated control. Data points are means ± the standard deviations of duplicate measurements and are representative of a minimum of three independent
experiments. Single (*) and double (**) asterisks indicate statistical significances of
statistical significances of $P < 0.01$ and $P < 0.0001$, respectively. (D) WT HMPV was treated with vehicle (untreated) or a K5 polysaccharide derivative at 1 μM and added to BEAS-2B cells at an MOI of 1 at 4°C for particle binding. Lysates of washed cells were analyzed
for HMPV binding by Western blotting for M. No virus was added to mock-infected cells. -Actin served as a loading control. (E) Band intensities of the matrix
protein and -actin were determined for untreated and treated (1 μM) samples. The data are reported as a ratio of M to -actin normalized to the untreated
control for each virus. Data points are means ± the standard deviations of measurements representative of five independent experiments. *, Statistical
significance of $P < 0.001$; **, statistical significance of $P < 0.0001$. (F) BEAS-2B cells were treated with 40 μg of iota-carrageenan/ml, 10 μM concentrations of
each of the K5 polysaccharide derivatives, or vehicle (untreated) in triplicate and assayed for viability by using an MTT cell viability assay according to the
manufacturer’s protocol. The absorbance at 590 nm was normalized to the untreated control. Data points are means ± the standard deviations of triplicate
measurements and are representative of three independent experiments.
The reduction of infection by the K5 polysaccharide was not expected, and the mechanism of this action remains unclear since it did not affect HMPV binding (Fig. 4D and E). None of the K5 polysaccharide derivatives had an effect on PIV5 infection (Fig. 4C). In addition, treatment of BEAS-2B cells with a 10 μM concentration of the K5 polysaccharide derivatives, the concentration used in HAE infection experiments and 10-fold higher than the highest concentration used in cell infection assays, did not reduce cell viability, as measured by an MTT cell viability assay (Fig. 4F). Similarly, treatment of BEAS-2B cells with the highest concentration of iota-carrageenan tested in the infection assays did not reduce cell viability, demonstrating that its antiviral activity is not a consequence of cytotoxicity (Fig. 4F). To determine whether the K5 compounds inhibit HMPV infection by competition, the binding assay described in the carrageenan studies was used. rgHMPV was treated with a 1 μM concentration of each of the K5 polysaccharide derivatives prior to incubation with BEAS-2B cells at 4°C at an MOI of 1 to allow for binding to take place. Although unmodified K5 and K5-NS, which have a single N-linked sulfation modification, had no effect on viral binding, the higher-sulfated compounds, K5-N,OS(L) and K5-OS(L), and the highly sulfated compounds K5-N,OS(H) and K5-OS(H) reduced HMPV binding to BEAS-2B cells significantly (Fig. 4E).

To confirm these findings in a physiologically relevant tissue model, we determined the effect of K5-N,OS(H) and K5-OS(H), which had the greatest inhibition of HMPV infection in monolayer cells, in polarized airway tissues. HAE tissues were infected at the apical surface with rgHMPV or rgPIV5 at an MOI of 5 treated with 10 μM K5-N,OS(H) or K5-OS(H), or with vehicle (untreated) and imaged at 48 h postinfection at ×5 magnification. (B) Quantification of HAE tissue infection. Data points are means ± the standard deviations of triplicate measurements and are representative of a minimum of three independent experiments. *, Statistical significance (P < 0.0001).

**FIG 5** Highly sulfated K5 polysaccharide derivatives inhibit HMPV infection in HAE. (A) HAE tissues were infected with rgHMPV or rgPIV5 at an MOI of 5 treated with 10 μM K5-N,OS(H) or K5-OS(H) or with vehicle (untreated) and imaged at 48 h postinfection at ×5 magnification. (B) Quantification of HAE tissue infection. Data points are means ± the standard deviations of triplicate measurements and are representative of a minimum of three independent experiments. *, Statistical significance (P < 0.0001).
Further ligand binding are less likely to disrupt preexisting HS interactions and therefore serve as an alternative approach to address the direct interaction of HMPV with HS. Furthermore, a compound that occludes HS and inhibits HMPV infection may serve as a potential building block for antiviral development for HMPV and other viruses that are known to bind HS.

To accomplish this, we utilized a previously characterized heparan sulfate occluding compound, peptide dendrimer SB105-A10 (54, 61, 62). Peptide dendrimers, branched synthetic molecules that consist of a peptidyl branching core and covalently attached surface peptide units, have a number of potential applications, especially in relation to the development of antiviral agents. The peptide dendrimer SB105-A10 (Fig. 1C), which has a branched peptide core with clusters of basic residues that bind to negatively charged sulfate and carboxyl groups, has been shown to specifically occlude ligand binding from HSFGs (61, 62). Further-
more, SB105-A10 has previously been reported to exhibit antiviral activity against RSV (54), CMV (61), HIV (62), HPV (63), and HSV-1 and HSV-2 (64), as well as some filoviruses (65).

To determine whether SB105-A10 reduces HMPV infection in human lung epithelial cells, BEAS-2B cells were treated with SB105-A10 at 1 μM prior to infection with rgHMPV at an MOI of 1, and the cells were imaged 24 h later for GFP expression; rgPIV5 was used in control studies to determine specificity. SB105-A10 treatment resulted in dramatic inhibition of HMPV infection, whereas PIV5 infection was not reduced (Fig. 6A). We performed quantification of the effects of SB105-A10 on rgHMPV or rgPIV5 infection by flow cytometry for GFP expression 24 h postinfection. In these experiments, both BEAS-2B and A549 cells were used to determine whether the effect of SB105-A10 is cell type- dependent, since this compound is mediating its effects by interacting with the target cell. In BEAS-2B cells, a dose-dependent inhibition of approximately 70% of rgHMPV infection resulted with SB105-A10 treatment, whereas rgPIV5 infection was not affected (Fig. 6B). Similar results were seen in A549 cells (Fig. 6C). In addition, treatment of BEAS-2B cells 2 μM SB105-A10, the highest concentration used in any cell or tissue experiments, did not reduce cell viability, as measured by an MTT cell viability assay (Fig. 6F). Based on our hypothesis of HMPV attachment, we predicted that SB105-A10 inhibits infection by blocking particle binding, specifically by preventing the interaction between heparan sulfate and F. To address this, we utilized a binding assay with WT HMPV, ΔG HMPV, and ΔGΔSH HMPV to determine the effects of SB105-A10. BEAS-2B cells were treated with 1 μM SB105-A10 or Opti-MEM prior to the addition of HMPV at an MOI of 1. The cells were incubated at 4°C for 2 h to allow for binding, and then cell lysates were analyzed for M by Western blotting to determine binding. A significant reduction in viral binding was observed in WT HMPV and the recombinant ΔG HMPV and ΔGΔSH HMPV with SB105-A10 treatment (Fig. 6D and E). As was observed in binding assays with iota-carrageenan (Fig. 2D), greater baseline binding was observed for ΔG HMPV and ΔGΔSH HMPV compared to the WT (Fig. 6D), although the same levels of reduction in binding were observed with SB105-A10 (Fig. 6E).

To determine the effect of SB105-A10 in polarized tissues, the apical surfaces of HAE tissues were treated with SB105-A10 at 2 μM prior to infection with rgHMPV or rgPIV5 at an MOI of 5. Treatment with SB105-A10 resulted in a reduction of HMPV infected cells 48 h postinfection, whereas PIV5 infection was not inhibited (Fig. 7A). Quantification of infected cells revealed a >50% reduction in HMPV infection at the apical surface with SB105-A10 treatment compared to vehicle-treated control tissues (Fig. 7B). Altogether, these results indicate that the occlusion of HS moieties on target cells inhibits HMPV binding and infection mediated by HMPV F and further support that HS is available for viral binding at the apical surface of the airway. Furthermore, based on our results, occlusion of HS could potentially be used as an antiviral strategy against HMPV.

DISCUSSION

Heparan sulfate is a key attachment factor for HMPV binding to the cell surface. In this study, we used compounds that modulate the attachment event to characterize the interaction between HMPV and HS. Our results support a model in which HMPV F mediates a direct binding interaction to HS, which can be inhibited by sulfated polysaccharides, specifically sulfated in the O position, and HS occluding compounds (Fig. 8). Whether these approaches can be used simultaneously to achieve greater inhibition of HMPV infection synergistically remains to be determined. Our results further indicate that HS in the airway epithelium serves as a binding factor during infection at the apical surface and suggest that HS modulating compounds may serve as a platform for potential HMPV antiviral development.

Iota-carrageenan treatment of HMPV resulted in inhibition of attachment (Fig. 2D) and infection (Fig. 2A and C) in bronchial epithelial cells and polarized airway tissues (Fig. 2F). The anti-HMPV activity of a sulfated polysaccharide has been previously reported using native and depolymerized galactans isolated from the red seaweed Cryptonemia seminervis (66). Although iota-carrageenan is a highly heterogeneous polysaccharide with regard to size, it is unclear whether its molecular mass is important in the inhibition of the viral interaction with heparan sulfate. It has been shown that depolymerized galactans ranging in molecular masses from 52 to 64 kDa were able to inhibit HMPV infection, as well as the intact polysaccharide, suggesting that low-molecular-mass sulfated polysaccharides can have potent antiviral activity (66). The potent anti-HMPV effect of iota-carrageenan on HMPV in-

FIG 7 Treatment of HAE tissues with SB105-A10 reduces HMPV infection. (A) HAE tissues were treated with 2 μM SB105-A10 or vehicle (untreated) and infected with rgHMPV or rgPIV5 at an MOI of 5. Tissues were imaged at 48 h postinfection at ×5 magnification. (B) Quantification of HAE tissue infection. Data points are means ± the standard deviations of triplicate measurements and are representative of a minimum of three independent experiments. *, Statistical significance (P < 0.0001).
our results highlight that variations in the structure of the K5 derivatives, namely, the position and degree of sulfation, can modulate the selectivity and potency of their activities against other HS-binding viruses, including hepatitis C virus (72), dengue virus, and the two main protein families with HS are syndecans and glypicans. Syndecans have been shown to serve as receptors for viruses in the respiratory system, including RSV, which has entirely different sulfation modifications in the human airway. HS occlusion with SB105-A10 has also been shown to inhibit RSV infection at the apical surfaces of HAE tissues (54). HS content in the recombinant HMPV viruses did not reveal a greater antiviral activity. Furthermore, it has been shown RSV has entirely different sulfation requirements for binding to HS, since N-sulfation is required, whereas O-sulfation is dispensable (68), demonstrating that the binding between viruses and surface HSs are very specific.

Interestingly, binding experiments in the present study demonstrated a greater affinity of ΔG and ΔG\text{ASH} HMPV to bind BEAS-2B cells than WT HMPV (Fig. 2D and 6D), since the untreated control bands consistently showed higher levels of particle binding for the recombinant viruses compared to the WT, despite equivalent number of particles added to the cells. Analysis for F content in the recombinant HMPV viruses did not reveal a greater F content relative to the M viral protein (Fig. 3D). We have previously reported that the ΔG and ΔG\text{ASH} recombinant viruses bind and infect at WT levels in other cell types, suggesting that there may be cell-type-specific differences in binding. Taken together, these results suggest that SH and G negatively modulate binding in BEAS-2B cells, and thus their absence results in more efficient particle binding. Both HMPV SH and G have been previously reported to modulate events in HMPV entry. Our group has shown SH can modulate fusion activity of F (69). Furthermore, HMPV G and SH have been previously shown to negatively modulate HMPV entry, as particle uptake by micropinocytosis in dendritic cells is enhanced for recombinant HMPV lacking G and SH (70). Soluble HMPV G has been shown to bind HS in vitro (71), which suggests that it may have some interaction with HS at the surfaces of cells and the apical surfaces of HAE cultures. However, results in monolayer cells suggest that HMPV F is the principal driver of particle interaction with HS (Fig. 2D). Further studies with recombinant HMPV lacking G and/or SH are necessary to determine what role these proteins may play during the critical early steps in HMPV infection of cell culture and HAE model systems.

Treating the cells and tissues with SB105-A10, which specifically occludes any ligand binding to HS PGs, resulted in a significant inhibition of HMPV binding and infection. Our results further support the model that HMPV uses HS as an attachment site due to a direct binding interaction with F. While adhered immortalized cells readily express accessible heparan sulfate, it is less clear where HS localizes in the respiratory epithelium in vivo. Based on detection by immunohistochemistry of human epithelial tissue, heparan sulfate has been previously hypothesized to localize exclusively to the basolateral epithelium (50), making it unclear how a respiratory virus would access HS to infect apically. The results in this study demonstrate that HMPV can infect polarized airway tissues at the apical surface and that HS occlusion inhibits this apical infection, suggesting that HS is found at sufficient levels to promote attachment at the apical surface of the airway. HS occlusion with SB105-A10 has also been shown to inhibit RSV infection at the apical surfaces of HAE tissues (54). HS modification is found on a number of transmembrane proteins, and the two main protein families with HS are syndecans and glypicans. Syndecans have been shown to serve as receptors for other HS-binding viruses, including hepatitis C virus (72), dengue virus (73), and HIV (30). Anti-syndecan-1 antibodies have recently been shown to block RSV infection at the apical surface of human airway epithelium cultures (74). The role of a specific HS PG, such as one of the syndecan proteins, in HMPV infection...
remains to be determined, but our results strongly indicate that sufficient levels of H5 on HSPGs are exposed at the apical surface of the airway epithelium for viral infection, including infection with HMPV.

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