Online Data Supplement

RHINOVIRUS DISRUPTS THE BARRIER FUNCTION OF POLARIZED AIRWAY EPITHELIAL CELLS

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METHODS

Viruses. Rhinovirus serotypes 16 (RV16), 39 (RV39) and 1B (RV1B) were purchased from American Type Culture Collection (Manassas, VA) and viral stocks were generated as described previously (E1). Briefly H1 HeLa cells were infected with RV until 80% of the cells were cytopathic. Hela cell supernatants were harvested, subjected to two freeze-thaw cycles and centrifuged at 10, 000 x *g* for 30 min at 4°C to remove cellular debris. RV was concentrated and partially purified by ultrafiltration through 100,000 MW cutoff filter using an Amicon stir cell module (Millipore, Billerica, MA) . Similarly-treated HeLa cell supernatants from uninfected cells served as controls (sham infection). Tissue culture adapted influenza A virus was purchased from ATCC. Stocks of influenza A virus was generated by infecting MDCK cells as suggested by the provider. Respiratory syncitial virus (RSV) was kindly provided by Dr. N. Lukacs, (University of Michigan). RSV subtype A, Umich/line 19 strain was derived from a clinical isolate at the University of Michigan and was propagated in Hep2 cells. Infection was allowed to proceed until syncytia were observed. Cells were frozen at –80°C and the supernatant was harvested, clarified, and aliquoted. Fifty percent tissue culture infectivity ($TCID_{50}$) values of viral stocks were determined by the Spearman-Karber method (E2).

Bacteria and growth conditions. Clinical isolates of NTHi (6P5H, 5P19H1 and 45P9H1) were obtained from COPD patients at the time of exacerbation and kindly provided by Dr. T. Murphy (University of Buffalo). *P. aeruginosa*, strain PAO1 was kindly provided by Dr. Goldberg, University of Virginia, Charlottesville). *Staphylococcus aureus* (Wood 46) was from American Type Tissue Culture. All bacterial stocks were maintained as glycerol stock at -80°C. For infection assays, all NTHi isolates were subcultured on chocolate agar plates and incubated overnight at 37°C/5% CO_2. *P. aeruginosa* and *S. aureus* were cultured on brain heart

infusion agar and nutrient agar, respectively, and grown overnight at 37°C. Bacteria were scraped off of the plate, suspended in serum and antibiotic-free cell culture medium to the required concentration.

Cell culture. Human primary airway epithelial cells obtained from the tracheal trimmings of donor lungs at the time of double lung transplantation were cultured in collagen coated plates using bronchial epithelial cell culture media (BEGM) (Cambrex Bioscience, Walkersville, MD), as previously described (E3). Cells were stored in liquid nitrogen until required. To differentiate cells into a mucociliary phenotype, passage one epithelial cells were seeded on collagen-coated transwells and grown under submerged conditions in BEGM until the cells were confluent. Cells were then shifted to air/liquid interface and maintained in a 1:1 mixture of BEGM and Dulbecco's modified Eagle medium (DMEM) for two weeks.

16HBE14o- human bronchial epithelial cells originating from bronchial epithelial tissue transfected with pSVori-, containing the origin-defective SV40 genome (E4), were also studied. Cells were grown in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 2 mM of L-glutamine. For all assays cells were grown in Tranwells with 0.4 or 3 m pore size semipermeable membranes and fed from both the basolateral and apical sides. Cells were maintained in culture until the transepithelial resistance (R_T) reached 800 to 1000 $\Omega.cm^2$.

Calu-3 human lung adenocarcinoma cells were purchased from ATCC and cultured in DMEM containing 10% FBS, 2mM of L-glutamine. Cells were grown in Transwells as described above until the cells attained R_T of 1500 to 1900 $\Omega.cm^2$.

Infection of cell cultures and measurement of transepithelial resistance (R_T). To calculate multiplicity of infection (MOI), for each experiment, we counted the cells in an

identically seeded well. Then, based on the number of cells and the $TCID_{50}$ of virus, we adjusted the virus concentration. For instance, when cells are confluent in a 12 mm transwell, the cell number usually falls around 2 x 10^6 cells/well. In this case, we adjust the concentration of virus to 4 x 10^6 $TCID_{50}$/ml, and use 0.5 ml to infect the cultures, leading to an approximate MOI of 1.0. On the day of infection, medium in the basolateral chamber was replaced with fresh antibiotic-free medium, and the apical surface was infected with 50 μl RV39, multiplicity of infection (MOI) of 1, 2, 5 or 10, or an equivalent volume of sham control. After 1 h (16HBE14o- and Calu-3 cells) or 5 h (primary airway epithelial cells) of incubation, medium from the apical surface was removed and the cells were further incubated for 24 or 48 h at 33°C. In selected experiments, 16HBE14o cells were infected with RSV or influenza A virus (MOI, 1) and the experiment continued as described above. Media in the apical chamber was changed and R_T was measured with an EVOM voltmeter equipped with EndOhm 6 tissue resistance measurement chamber (World Precision Instruments, Sarasota, FL) (E5).

Paracellular permeability. 16HBE14o- cells were infected with RV39 at MOI of 1 as described above. Twenty-four h later, 100 μl of FITC-inulin (1 mg/ml) was added to the apical chamber and further incubated for 6 h. Media from both apical and basolateral chambers was sampled at 15, 30, 45 and 60 min, 2, 3, and 6 h, and the amount of fluorescence was measured in a microtiter plate reader (Molecular Devices, Sunnyvale, CA). The paracellular permeability of polarized epithelium to FITC-inulin from the apical to the basolateral chamber (P_{app}) was calculated as described previously (E3, E6).

Nonidet P-40-soluble and -insoluble cell extracts. After the specified treatments, cells were rinsed with ice-cold saline (pH 7.4), and incubated for 15 min on ice with 100 μl of Nonidet P-40 (NP-40) solubilization buffer [25 mM HEPES (pH 7.4), 150 mM NaCl, 4 mM

EDTA, 25 mM NaF and 1 mM Na_3VO_4 with 1% (vol/vol) NP-40 and Complete Protease Inhibitor (Roche Diagnostics, Indianapolis, IN)]. NP-40-insoluble material was pelleted (14,000 *g* for 10 min at 4°C), the supernatant was saved, and Laemmli reducing buffer was added to the pellet, which was then heated for 10 min at 100°C. Protein concentration in NP-40 soluble fraction was determined by Lowry assay (BioRad, Hercules, CA).

Western blot analysis. NP-40-soluble and -insoluble fractions (equivalent to 50 μg of total protein) were separated by SDS-PAGE and proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with TBS-T [10 mM Tris (pH 7.4), 150 mM NaCl and 5 mM KCl with 0.05% (vol/vol) Tween 20] containing 5% skim milk. Blots were incubated over night at 4°C with antibodies to zona occludins (ZO)-1or β-actin (Invitrogen, Carlsbad, CA). Blots were washed and incubated with species-appropriate peroxidase-conjugated secondary antibodies (Invitrogen), washed with TBS-T, and developed using a chemiluminescence system (Super Signal, Pierce, Rockford, IL). Specific bands were quantified by densitometry using NIH imageJ.

Confocal indirect immunofluorescence. After appropriate treatments, well-differentiated airway epithelial cell cultures were washed with PBS, fixed in cold methanol for 5 min at -20°C, and permeabilized with 0.1% (vol/vol) Triton X-100. Cells were blocked with PBS containing 1% (wt/vol) bovine serum albumin (BSA) for 1 h and incubated overnight at 4°C with mouse primary polyclonal antibody to ZO-1 (1 μg/ml) (BD Biosciences, San Jose, CA) alone or in combination with antibody to heat-killed NTHi (provided by Dr. Graham Krasan, University of Michigan, Ann Arbor) . Bound antibodies were detected by using Alexa Flour-conjugated second antibody and visualized by confocal fluorescent microscopy with a Zeiss LSM 510 confocal microscope mounted on a Zeiss Axiovert 100 M inverted microscope. Optical sections

were taken through the cell monolayers at 0.2 μ intervals. XZ sections were constructed using LSM image browser software (Carl Zeiss Microimaging, Thornwood, NY). Cells treated with normal IgG instead of primary antibody served as negative controls. For paraffin sections, 5-8 μ thick sections were deparaffinized, heat-treated to expose antigenic sites, blocked with 5% normal goat serum and then incubated with rabbit polyclonal antibody to ZO-1 (Invitrogen, Carlsbad, CA). Bound antibody was detected as described above.

Transmission electron microscopy. Polarized 16HBE14o- cells were infected apically with RV at MOI of 1 or equal volume of sham as described above. Next day cells were super infected with 0.1 ml of 1 x 10^9 CFU/ml NTHi 6P5H and incubated for 3 h. Cells were rinsed in serum free medium and then fixed for one hour at room temperature in 2.5 percent glutaraldehyde in 0.1 M Sorensen's buffer, pH 7.4. Following a buffer rinse, they were postfixed for 15 minutes in 1% osmium tetroxide, washed with water and stained with saturated solution of uranyl acetate in water. They were then dehydrated rapidly in a graded series of ethanol, infiltrated and embedded in Epon, and polymerized. Ultra-thin sections were collected onto Formvar/carbon-coated, slotted copper grids and post stained with uranyl acetate and lead citrate. The sections were viewed on a Philips CM100 at 60 kV. Images were recorded digitally using a Hamamatsu ORCA-HR digital camera system, which was operated using AMT software (Advanced Microscopy Techniques Corp., Danvers, MA).

Detection of apoptosis. Polarized 16HBE14o- cells were treated with thapsigargin (2 or 5 μM for 24 h), and apoptotic, non-necrotic cells detected by flow activated cell sorting with FITC-conjugated annexin V and propidium iodide as described previously (E7) (Apoptosis Detection Kit, Sigma Chemical, St. Louis, MO).

Inoculation of mice. C57BL/6 mice were inoculated intransally with RV1B ($TCID_{50}$ of 5

x 10^7 per mouse) or equal volume of sham, as described (E8). Twenty four h later, mice were sacrificed and the lungs were perfused with 20 mM EDTA, inflated and fixed in buffered formalin, and embedded in paraffin.

Data analysis. Statistical significance was assessed by analysis of variance (ANOVA). Differences identified by ANOVA were pinpointed by Tukey-Kramer test.

RESULTS

RV infection does not cause reductions in R_T by inducing cytotoxic effects: trypan blue studies. To examine whether RV induces cytotoxicity in primary human mucociliary-differentiated airway epithelial cells or polarized 16 HBE14o- cells, we infected these cells with major or minor group of RV and incubated for 24 h. Cells were dissociated from the membrane and incubated with 2% trypan blue for 3 min at room temperature. Cells were counted under microscope to estimate the percentage cells positive for trypan blue (Figure). None of the RV-infected primary cell cultures showed significant increase in number of the dead cells. Similar results were observed with 16HBE14o- cells except when cells that were infected with RV1B at 5 MOI, which showed a slight increase in the percentage of dead cells.

REFERENCES

E1. Newcomb DC, Sajjan U, Nanua S, Jia Y, Goldsmith AM, Bentley JK, Hershenson, MB. 2005. Phosphatidylinositol 3-kinase is required for rhinovirus-induced airway epithelial cell interleukin-8 expression. *J. Biol. Chem.* 280: 36952-36961.

E2. Johnston SL, Tyrrell DAJ, Rhinoviruses In: Lennette EH. 1997Schmidt NJ eds. In Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections Washington D.C: American Public Health Association. pp. 533-563.

E3. Sajjan U, Keshavjee S, Forstner J. 2004. Responses of well-differentiated airway epithelial cell cultures from healthy donors and patients with cystic fibrosis to Burkholderia cenocepacia infection. *Infect. immun.* 72: 4188-4199.

E4. Cozens AL, Yezzi MJ, Kunzelmann K, Ohrui T, Chin L, Eng K, Finkbeiner WE, Widdicombe JH, Gruenert DC.1994. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol*. 10: 38-47.

E5. Kim JY, Sajjan US, Krasan GP, LiPuma JJ. 2005. Disruption of tight junctions during traversal of the respiratory epithelium by *Burkholderia cenocepacia. Infect. immun.*;73: 7107-7112.

E6. Coyne CB, Vanhook MK, Gambling TM, Carson JL, Boucher RC, Johnson LG. 2002. Regulation of airway tight junctions by proinflammatory cytokines. *Mol. Biol. Cell* 13: 3218-3234.

E7. Nanua S, Sajjan U, Keshavjee S, Hershenson MB, 2006. Absence of typical unfolded protein response in primary cultured cystic fibrosis airway epithelial cells. *Biochem Biophys. Res. Commun*. 343: 135-143.

E8. ` Newcomb DC, Sajjan US, Nagarkar DR, Wang Q, Nanua S, Zhou Y, McHenry CL, Hennrick KT, Tsai WC, Bentley JK, et al. Human rhinovirus 1b exposure induces pi 3-kinase-dependent airway inflammation in mice. *Am J Respir Crit Care Med* 2008: 177:1111-21.

Figure 1 on-line supplement