Rhinovirus-A1B decreases coronavirus NL63 replication and inflammation in adult mice.

J. Kelley Bentley, Haley A. Breckenridge, Jordan E. Kreger, Adam M. Goldsmith, Carey N. Lumeng, and Marc B. Hershenson.

Dept. of Pediatrics, University of Michigan Medical School, Ann Arbor, Michigan

ABSTRACT

Introduction: SARS-CoV2 produces a range of illness ranging from asymptomatic to acute respiratory failure. One disease factor is the host interferon response. Data suggest SARS-CoV2 pathology is modulated by previous respiratory viral infections. We examined whether prior rhinovirus-A1B infection modulates infection with the a-human coronavirus NL63 (HCoV-NL63). **Methods:** HCoV-NL63 (BEI Resources) propagated in HeLa cells stably expressing human ACE2. RV-A1B (ATCC) was grown in HeLa-H1 cells. Adult (6-8 week) C57BL6/J or B6.Cg-Tg(K18-ACE2)2Prlmn/J mice expressing hACE2 protein in airway epithelial cells were infected intranasally with sham cell lysate or 1 x 10^6 PFU RV-A1B in 50 mL PBS. Four days later, mice were infected with HCoV-NL63 (1 x 10^5 $TCID_{50}$ units). At 3 or 5 days post-HCoV-NL63 infection, mouse lungs were assessed for HCoV-NL63 vRNA, broncheoalveolar lavage (BAL) cells, and histology. Cultured primary human bronchial epithelial cells (HBEs, Lifeline Cell Technology) were mucociliary-differentiated on Transwell membranes (Corning) at air-liquid interface for 3-12 wk in PneumaCult medium (StemCell Technologies). Cells were infected with sham HeLa cell lysate or 1 MOI RV-A1B and, 48 h later, treated with sham or 0.1 MOI HCoV-NL63. Cells were harvested at 6, 24, and 48 h post-NL63 infection and processed for RNA. **Results**: Three days after HCoV-NL63 infection, HCoV-NL63 copies were higher in sham hACE2 mice (hACE2, 2791±1593 copies/µg RNA; C57BL6/J, 16±13 copies/µg RNA; N=4, mean ± SEM, p=0.0017, ANOVA). Prior RV-A1B significantly decreased HCoV-NL63 copy number in hACE2 mice (15±3 copies/µg RNA, p=0.0017). Results at five days after HCoV-NL63 were similar. Infection of hACE2 mice with HCOV-NL63 caused perivascular and peribronchial inflammation. Three days after HCoV-NL63, airway neutrophils and lymphocytes were significantly higher in hACE2 mice compared to wild-type mice (neutrophils hACE2 1.95 ± 0.64 x 10^5 cells/lung, C57BL/6 3.38 ± 1.25 x 10^3 cells/lung, p<0.0013; lymphocytes hACE2 1.02 ± 0.22 x 10^5 cells/lung, C57BL/6 2.750 ± 1.7 x 10^3, p<0.0001, ANOVA). Prior RV-A1B infection significantly reduced neutrophils and lymphocytes in HCoV-infected hACE2 mice (neutrophils, 0.34 ± 0.18 x 10^3 cells/lung, p=0.003 and 1.56 x ± 0.76 x 10^4 cells/lung, p<0.0001). In human bronchial epithelial cells, HCoV-NL63 vRNA levels peaked 24-48 h after infection. Prior RV-A1B infection significantly decreased HCoV NL63 replication (vRNA 24 h infection sham/HCoV-NL63 infected wells, 4809±3612 copies/µg RNA; RV-A1B/HCoV-NL63-infected wells, 93±66 copies/µg RNA, n=4, p=0.0003). Supported by National Institutes of Health grants AI120526 and AI155444.

INTRODUCTION

Recent data suggest human rhinovirus act to block SARS-CoV2 infection in the epithelium (1,2). Like SARs CoV2, alpha coronavirus NL63 (HCoV-NL63) causes respiratory infections in humans and shares the same receptor (angiotensin-converting enzyme 2, ACE2). NL63 has been sequenced in its entirety (3), allowing the development of a viral copy number assay that along with titration for cytopathic effect can be used to quantitate the virus. Antibodies have been developed to the viral nucleoprotein (4) as well as nonstructural proteins expressed during its replicative cycle intracellularly (5) that allow its detection in cells and tissues.

Using a transgenic mouse that expresses the human ACE2 receptor (5) and human bronchial epithelial cell culture, we find that human rhinovirus A1B lowers subsequent HCoV-NL63 replication.

METHODS

Virus propagation and titer. HCoV-NL63 (BEI Resources) was grown in Hela or LLC-MK2 transfected with a human ACE2 receptor (pLV[Exp]-mCherry:T2A:Puro-CMV>hACE2[NM_021804.3] , VectorBuilder, Chicago IL) using methods described (7,8). Initial stocks were prepared by lysing 3-day infected cells with trituration and centrifugation for 30 min at 10,000 x*g* to remove particles. Cleared cell supernatants from large scale preparations was concentrated by ultracentrifugation for 2h at 100,000 x*g*. Titer was determined on 96 well plates of LLC-MK2 cells at 80% confluence. Cytopathic effect visible after 48 h at 33ºC was verified using immunofluorescence with an antibody to nucleoprotein to estimate tissue culture infectious dose (TCID50). ***Viral detection.*** To detect HCoV-NL63 in cells and lung tissue, antibodies to the HCoV-NL63 N-protein and nsp3 (Genscript, Piscataway, NJ) were used. Purified primary antibodies were labeled for immunofluorescence using AlexaFluor dye-conjugated N-hydroxy succinimidyl esters or visualized by secondary immunofluorescence with goat- anti-rabbit IgG labelled with AlexaFluor dyes. Double-stranded RNA was detected by monoclonal rJ2 (Sigma, St. Louis MO) and secondary fluor-labelled goat anti-mouse IgG. ***Measurement of HCoV-NL63 infectivity: quantitative polymerase chain reaction (qPCR).*** To measure lung viral RNA, total RNA was prepared using Trizol (InVitrogen, Carlsbad, CA) extraction and RNAeasy purification (Qiagen). First strand cDNA was prepared using the SuperScript IV system (Applied Biosystems). RNA was detected with qPCR using Sybr Green (Applied Biosystems) extending from bases 24-106 of the GenBank sequence NC_005831.2 (9) . A plasmid containing bases 1-540 of the sequence was used as a copy number standard (GenScript, Piscataway, NJ). ***Genotyping mice.*** Mice (B6.Cg-Tg(K18-ACE2)2Prlmn/J, JAX Labs, Bar Harbor, ME) were genotyped using the recommended TaqMan qPCR assay on genomic DNA (10) and confirmed by RNA expression of human ACE2 as assessed by RT-qPCR. ***Infection of mice.*** Adult K18-ACE2 mice were inoculated intranasally first with 1x10^6 pfu of RV-A1B (11) and 4 days later with 1 x 10^5 TCID50 equivalents NL63 or cell sham. At indicated days mice were harvested for lung histology or RNA isolation. ***Histology and immunofluorescence.*** After sacrifice, mouse right arteries were perfused with PBS + 5mM EDTA, the left lung tied off and excised for RNA or protein. The tracheas were intubated and perfused with formalin, and the lungs processed for paraffin sectioning. Sections were stained with hematoxylin and eosin (H&E) or immunofluorescence as described in the Figure legends. Lung mRNA expression was estimated by qPCR as described in the Figure legends.

METHODS AND RESULTS

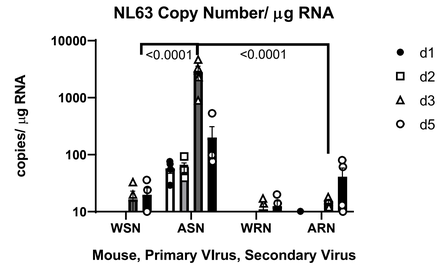

Figure 1. RV-A1B infection lowers HCoVNL3 replication in K18-ACE2 mice. After 4 days of RV-A1B (R) or sham (S), wild-type (W) or K18-ACE2 (A) mice were infected with HCoV NL63 (N) on day zero. On days 1 (d1, ●), 2 (d2, □), 3 (d3, △), or 5 (d5, ○) mouse lungs were harvested for RNA production and copy number assay. RV-A1B treatment significantly (p<0.001, Tukey's multiple comparison test, ANOVA) lowered the replication of HCoV-NL63.

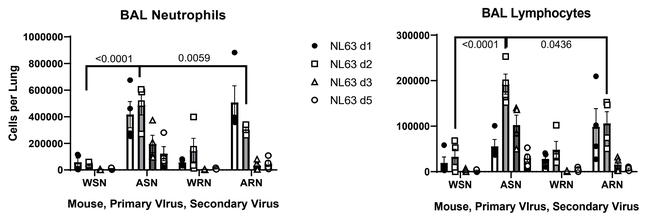

Figure 2. RV-A1B infection lowers the neutrophil and lymphocyte airway cell count in HCoVNL3-infected K18-ACE2 mice. After 4 days of RV-A1B (R) or sham (S), wild-type (W) or K18-ACE2 (A) mice were infected with HCoV NL63 (N) on day zero. On days 1 (d1, ●), 2 (d2, □), 3 (d3, △), or 5 (d5, ○) mouse lungs were harvested for broncheoalveolar lavage. RV-A1B treatment significantly (p values from Tukey's multiple comparison test, ANOVA) lowered the neutrophil and lymphocyte count after HCoV-NL63 infection.

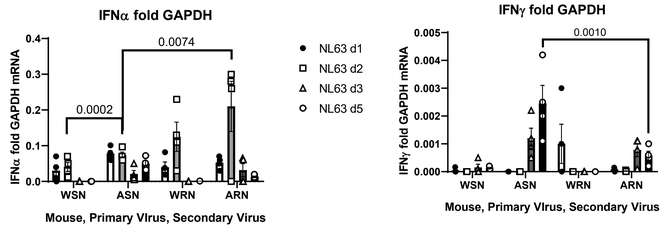

Figure 3. RV-A1B infection lowers the neutrophil and lymphocyte airway cell count in HCoVNL3-infected K18-ACE2 mice. After 4 days of RV-A1B (R) or sham (S), wild-type (W) or K18-ACE2 (A) mice were infected with HCoV NL63 (N) on day zero. On days 1 (d1, ●), 2 (d2, □), 3 (d3, △), or 5 (d5, ○) mouse lungs were harvested RNA and qPCR. RV-A1B treatment significantly (p values from Tukey's multiple comparison test, ANOVA) increased the IFNa response on day 2 after HCoV-NL63 infection.

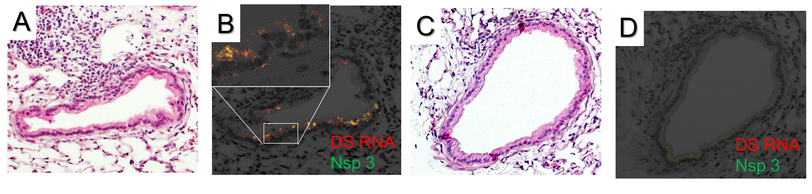

Figure 4. RV-A1B infection lowers the inflammation and detectable double-stranded RNA as well as nonstructural protein 3 in HCoVNL3-infected K18-ACE2 mice. After 4 days of sham (A,B), or RV-A1B, (C,D) K18-ACE2 mice were infected with HCoV NL63. On day 2 mouse lungs were harvested after inflation and formalin perfusion, and processed for paraffin sectioning. Shown in A is a medium sized airway from a sham-NL63 treated mouse stained with hematoxylin and eosin. In B, the same airway in a matching section is shown stained for double stranded RNA (DS RNA, red) and nsp3 (green). Shown in C is a medium sized airway from RV-A1B -NL63 treated mouse stained with hematoxylin and eosin. In D, the same airway in a matching section is shown stained for double stranded RNA (red) and nsp3 (green). RV-A1B treatment lowers the detectable double stranded RNA and nsp3 protein in the lung.

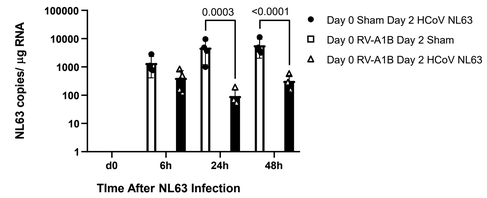

Figure 5. RV-A1B infection lowers the ability of HCoV-NL63 to replicate in cultured human bronchial epithelium. After 2 days of RV-A1B (1x10^6 pfu) or sham, cells were treated with sham again or 1x10^5 TCID units of HCoV NL63. Coronavirus copies were significantly higher at 24 and 48h post-infection in cells that were treated on day 0 with sham (●), than cells initially treated with RV1B (△), while undetectable in the double sham treated cells 2 (□), (p values from Tukey's multiple comparison test, ANOVA).

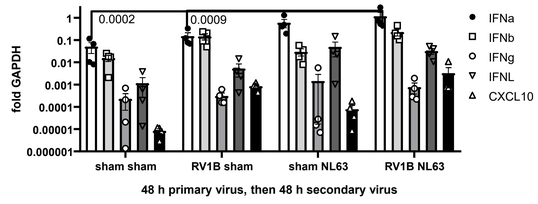

Figure 6. RV-A1B infection increases the ability of HCoV-NL63 to elicit an interferon response cultured human bronchial epithelium. After 2 days of RV-A1B (1x10^6 pfu) or sham, cells were treated with sham again or 1x10^5 TCID units of HCoV NL63. At 48h after HCoV NL63 infection, cells were harvested for RNA and qPCR to detect IFNα (●), IFNβ (□), IFNγ (○), IFNΛ (▽), or CXCL10 (△) transcripts and normalized to GAPDH (p values from Tukey's multiple comparison test, ANOVA).

SUMMARY AND CONCLUSIONS

HCoV NL63 infects K18-ACE2 mice and induces an airway inflammatory response.

Viral replication is evidenced by viral RNA levels, interferon mRNA expression and production of non-structural viral proteins. Treatment with RV-A1B may increase the interferon response to HCoV-NL63, and there interfere with HCoV NL63 replication in mice.

This model may be used to study HCoV-NL63-induced exacerbation of allergic airways disease; the effect of allergy, obesity and aging on human coronavirus infection, and possible cross-immunity between HCoV-NL63 and other respiratory viruses.

REFERENCES

1. Dee K, Goldfarb DM, Haney J, Amat JAR, Herder V, Stewart M, Szemiel AM, Baguelin M, and Murcia PR (2021*) J Infect Dis* 224(1):31-38.
2. Cheemarla NR, Watkins TA, Mihaylova VT, Wang B, Zhao D, Wang G, Landry ML, Foxman EF (2021) *J Exp Med*. 218:**e20210583**.
3. van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, Wertheim-van Dillen PM, Kaandorp J, Spaargaren J, and Berkhout B. (2004) *Nat Med.* 10(4):368-73.
4. Sastre, P., Dijkman, R., Camuñas, A., Ruiz, T., Jebbink, M. F., van der Hoek, L., Vela, C., and Rueda, P. (2011). *Clin. Vac. Immunol.* 18(1), 113–118.
5. Chen, Z., Wang, Y., Ratia, K., Mesecar, A. D., Wilkinson, K. D., and Baker, S. C. (2007). *J. Virol.* 81(11), 6007–6018.
6. Oladunni FS, Park JG, Pino PA, Gonzalez O, Akhter A, Allué-Guardia A, Olmo-Fontánez A, Gautam S, Garcia-Vilanova A, Ye C, Chiem K, Headley C, Dwivedi V, Parodi LM, Alfson KJ, Staples HM, Schami A, Garcia JI, Whigham A, Platt RN 2nd, Gazi M, Martinez J, Chuba C, Earley S, Rodriguez OH, Mdaki SD, Kavelish KN, Escalona R, Hallam CRA, Christie C, Patterson JL, Anderson TJC, Carrion R Jr, Dick EJ Jr, Hall-Ursone S, Schlesinger LS, Alvarez X, Kaushal D, Giavedoni LD, Turner J, Martinez-Sobrido L, and Torrelles JB. (2020) *Nat Commun.* 11(1):6122.
7. Herzog P, Drosten C, and Müller MA. (2008) *Virol J.* 5:138.
8. Schildgen O, Jebbink MF, de Vries M, Pyrc K, Dijkman R, Simon A, Müller A, Kupfer B, and van der Hoek L. (2006) *J Virol Methods*.138(1-2):207-10.
9. Donaldson EF, Yount B, Sims AC, Burkett S, Pickles RJ, and Baric RS. (2008) *J Virol* 82: 11948-11957
10. McCray PB Jr, Pewe L, Wohlford-Lenane C, Hickey M, Manzel L, Shi L, Netland J; Jia HP, Halabi C, Sigmund CD, Meyerholz DK, Kirby P, Look DC, and Perlman S. (2007) *J Virol* 81(2):813-21.
11. Newcomb DC, Sajjan US, Nagarkar DR, Wang Q, Nanua S, Zhou Y, McHenry CL, Hennrick KT, Tsai WC, Bentley JK, Lukacs NW, Johnston SL, Hershenson MB. (2008) *Am J Respir Crit Care Med*. 177(10):1111-21.