Published in final edited form as:

J Allergy Clin Immunol. 2014 August ; 134(2): 332–341.e10. doi:10.1016/j.jaci.2014.01.029.

Effects of rhinovirus species on viral replication and cytokine production

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Abstract

Background—Epidemiological studies provide evidence of differential virulence of rhinovirus (RV) species. We recently reported that RV-A and RV-C induced more severe illnesses than RV-B, suggesting that the biology of RV-B might be different from RV-A or RV-C.

Objective—To test the hypothesis that RV-B has lower replication and induces lesser cytokine responses than RV-A or RV-C.

Methods—We cloned full-length cDNA of RV-A16, A36, B52, B72, C2, C15 and C41 from clinical samples, and grew clinical isolates of RV-A7 and B6 in cultured cells. Sinus epithelial cells were differentiated at air-liquid interface. We tested for differences in viral replication in epithelial cells after infection with purified viruses (10^8 RNA copies) and measured virus load by quantitative RT-PCR. We measured lactate dehydrogenase (LDH) concentration as a marker of cellular cytotoxicity, and cytokine/chemokine secretion by multiplex ELISA.

Results—At 24 hours post infection, virus load of RV-B (RV-B52, B72, or B6) in adherent cells was lower than that of RV-A or RV-C. The growth kinetics of infection indicated that RV-B types replicate more slowly. Furthermore, RV-B released less LDH than RV-A or RV-C, and induced

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lower levels of cytokines and chemokines such as CXCL10, even after correction for viral replication. RV-B replicates to lower levels also in primary bronchial epithelial cells.

Conclusions—Our results indicate that RV-B types have lower and slower replication, and lower cellular cytotoxicity and cytokine/chemokine production compared to RV-A or RV-C. These characteristics may contribute to reduced severity of illnesses that has been observed with RV-B infections.

Clinical implications—RV-B types replicate at a lower rate and produce less cytokine/chemokine compared to RV-A or RV-C, which may contribute to the clinical observation that RV-B causes less severe illnesses.

Capsule summary—RV-B types replicate more slowly and to lower levels, and less cytokine/chemokine production compared to RV-A or RV-C. These characteristics may contribute to reduced severity of illnesses that has been observed with RV-B infections.

Keywords

Asthma; cellular cytotoxicity; chemokine; cytokine; rhinovirus

INTRODUCTION

Acute respiratory infections commonly precede asthma exacerbation in both children and adults.^{1–3} Community-based studies have identified viral infections in 80–85% of asthma exacerbations, and found that human rhinovirus (RV) is involved in approximately 65% of patients in whom a causative virus was identified.^{2–5} It is well known that RVs have tremendous diversity.^{6–10} In addition to about 100 classical serotypes that belong to RV species A (RV-A) or B, over 60 types of RV-C were recently discovered by molecular techniques.^{6–9}

Recent clinical data suggest differential virulence of RV species.^{11–21} For example, we prospectively collected nasal samples from a cohort of infants, and found that RV-B caused less severe illness than RV-A or RV-C.¹⁶ All RV-B types that we detected were less virulent than any of the RV-A or RV-C types.¹⁶ Furthermore, most studies have suggested that RV-B is less commonly detected during colds or acute wheezing.^{12, 13, 15–18} However, RV-B is detected more often when samples are obtained at routine intervals regardless of the presence of symptoms (ref 16 and unpublished data), providing evidence that RV-B infections are prevalent but typically cause mild or no symptoms. Studies of more severe illness or asthma exacerbations have generally reported that RV-C^{11–15} or RV-C and RV-A^{16–20} are overrepresented. These findings suggest that the virus biology including replication and anti-viral response might also differ by species.

There is relatively little information about RV-C growth characteristics due to difficulties in culturing this species. We reported that RV-C15 grows well in organ cultures of human sinus epithelial cells (HSECs) obtained as a by-product of human sinus surgeries.²² Furthermore, RV-C types have also been grown in differentiated HSECs that were cultured at the air-liquid interface (ALI).^{23, 24} This technique has facilitated quantitative studies of RV-C biology.

To test the hypothesis that RV-B has reduced replication and induces a lesser inflammatory response than other RV, we cloned seven clinical isolates of RV and isolated two others in tissue culture, and compared effects of RV species on the viral replication, cellular cytotoxicity, and cytokine secretion using the HSEC ALI culture. We found RV-B types replicate more slowly and to lower levels compared to either RV-A or RV-C types. RV-B-infected cells released less lactate dehydrogenase (LDH), a marker of cellular cytotoxicity,²⁵ compared to those infected with RV-A or RV-C. Furthermore, RV-B induced lower levels of cytokines and chemokines such as CXCL10. These characteristics likely contribute to the clinical observation that RV-B causes less severe illnesses than other RV species.

METHODS

Viruses

RV-C15 is a clinical isolate that we cloned previously.²² RV-A16 is a clinical isolate that was cloned and provided by Dr. Wai-Ming Lee (Biological Mimetic Inc.). Furthermore, we cloned the full-length cDNA copies of RV-B52, RV-B72, RV-A36, and RV-C41 from clinical isolates. We also obtained the full-length cDNA of RV-C2 by gene synthesis Genewiz Inc., South Plainfield, NJ). All isolates were obtained from nasal secretion samples of infants participating in a birth cohort that was performed from 1998 to 2001.¹⁶ Methods for cloning and virus production by RNA transfection are provided in the Methods section in this article's Online Repository (strategy for RV-B52 cloning (Fig E1), RV-B72 cloning (Fig E2), RV-A36 cloning (Fig E3), and RV-C41 cloning (Fig E4), and virus-specific primers (Table E1)).

RV-A7 and RV-B6 are also clinical isolates that were produced by inoculating nasal secretions into WisL or Hela cells, as previously described,^{26, 27} and purified using the same protocol used with the cloned viruses except for RNase A treatment.

The University of Wisconsin Human Subjects Committee approved the protocol and informed consent was obtained from the patient's families.

Cultures of HSECs or primary bronchial epithelial cells (PBECs) grown at ALI and inoculation

We cultured HSECs or PBECs at ALI as previously described.^{23, 24, 28, 29} After complete differentiation of epithelial cells (2 months), we infected cells with purified viruses (10^8 RNA copies per well).²³ See the Methods section in this article's Online Repository for further details.

Quantitative (q) RT-PCR

Total RNA was extracted from epithelial cells using the RNeasy Mini kit (Qiagen, Valencia, CA). RV RNA concentrations were determined by qRT-PCR as previously described.^{22, 23}

Cellular cytotoxicity, tight junction permeability, and apoptosis

Basal medium after virus-infection was assayed for LDH release (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI). Caspase 3/7 activity was assessed

using Apo-ONE Homogeneous Caspase-3/7 Assay (Promega). Further details including tight junction permeability (transepithelial resistance (TER) and FITC-dextran transit) are provided in the Methods section of this article's Online Repository.

Cytokine/chemokine production

Basal medium was assayed for CCL5, CXCL8, CXCL10, CXCL11, IFN- α 2, IFN- β , IFN- λ 1, and IL-6 by multiplex (Milliplex, Millipore, Temecula, CA) or solitary ELISA. See the Methods section in this article's Online Repository for further details.

In vitro RNA transcription and transfection of WisL cells with RV RNA

In vitro RNA transcription and transfection were performed using previously reported methods.²² RNA transcripts were synthesized from plasmids using a RiboMax large-scale RNA production system T7 (Promega). WisL cells (in 12-well plates) were incubated with transfection medium (RNA and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) complexes) for 1 hour, which was then replaced with complete cell culture medium. After cell collection, three freeze and thaw cycles, and RNase A treatment, total RNA was extracted and RV RNA concentrations were measured. See the Methods section in this article's Online Repository for further details.

Statistical analysis

Values are expressed as means $\pm$ SEM. Groups were compared using paired *t*-tests and mixed-effects linear models with Tukey's adjustment for multiple comparisons. Z-scores for viral replication (adjusted for viral binding) and cytokines/chemokines (adjusted for viral replication) were obtained by regressing the outcome measure on the adjustment variable and extracting the standardized residuals. *P* values of $< .05$ were considered to indicate statistical significance.

RESULTS

RV-B types reveal lower viral binding, and lower and slower viral replication

We first examined species effects on viral replication. We infected differentiated HSECs with viruses (three types each of RV-A, RV-B, and RV-C; 10^8 RNA copies/sample) and then quantitated RV RNA by qRT-PCR just after infection (viral binding) and at 24 hours post infection (PI). As over 95% of RV RNA remained cell-associated at 24 hours PI (see Fig E5 in this article's Online Repository), we measured RV RNA in HSECs and compared it among RV species. Compared to RV-A or RV-C types, the amount of RV-B52, B72 or B6 RNA in HSECs was slightly lower just after infection (Fig 1, A), and these differences were more pronounced at 24 hours PI (Fig 1, B). When types in the same species were combined, amounts of RV-B RNA just after infection as well as at 24 hours PI were significantly lower than those of RV-A or RV-C RNA (Fig 1, C). Furthermore, the net increase in RNA (24 hours PI minus 4 hours PI) was lower in RV-B (Fig 1, D). These findings suggested that not only viral binding as assessed just after infection, but also viral replication as assessed at 24 hours PI, were lower in RV-B.

We examined the growth kinetics of infection using all nine viruses. The amounts of RV-A and RV-C RNA reached maximum at 24 or 48 hours PI (Fig 1, E). In contrast, RV-B RNA increased more gradually and reached maximum at 72 hours PI (Fig 1, E), indicating that RV-B types replicate more slowly *in vitro*. Compared to RV-A and RV-C, the amounts of RV-B types in HSECs at 72 hours PI were still significantly lower (Fig 1, E), and they did not increase after 72 hours PI (data not shown). Therefore, RV-B types replicated more slowly and to lower levels compared to RV-A or RV-C types.

We then compared the dose-related effects on virus replication among RV species. After inoculation with higher doses (10^6 RNA copies) of RV, the amount of cell-associated RV-B52 RNA was lower than that of RV-A16 RNA (Fig 2, A). At 24 hours PI, the amounts of RV-B52 RNA were lower than that of RV-A16 or C15 RNA at all doses tested (Fig 2, B). Furthermore, the net increase in viral RNA over 24 hours was less for RV-B52 compared to A16 and C15 (Fig 2, C), even after adjusting for differences in viral binding (Fig 2, D). These findings suggest that not only virus entry (e.g. receptor binding, endocytosis and uncoating) but also later stages of virus infection (e.g. replication, host shut off etc.) are impaired in RV-B types.

Effect of RV species on cellular cytotoxicity, barrier function, and apoptosis

Next we examined the effect of RV species on cytotoxicity in differentiated HSECs. As RV infection does not cause notable cytopathic effects in ALI cultures,²³ we assessed cellular cytotoxicity by measuring LDH concentrations in basal medium. We measured LDH release after inoculation with each of the nine RVs, and found that RV-B types released less LDH than RV-A or RV-C at 48 hours PI and 72 hours PI (Fig 3, A–C).

We also evaluated effects of RV infection on the barrier function of HSECs by measuring TER and FITC-dextran transit, and found no differences among RV species (data not shown). Furthermore, RV species induced similar levels of apoptosis, as measured by caspase 3/7 activity (Fig 3, D). Similar results were obtained when the cells were assessed at 8 or 48 hours PI (data not shown).

Transfection of WisL cells with RV-B52 produces less viral RNA than that with RV-A16 or C15

As described above, our findings suggest that after incubation of ALI cells with equivalent amounts of virus, RV-B types had lower binding and replication (Fig 1, C and D). To further confirm whether the lower RV-B replication was independent of receptor binding, we transfected WisL cells (fetal lung fibroblasts) with RNA from different RV species. Transfection of RV-B52 RNA produced less virus at 16 hours post transfection as compared to that of RV-A16 or C15 RNA (Fig 4, A and B). In addition, transfection of RV-B52 RNA caused less cytopathic effect than that of RV-A16 or C15 RNA (Fig 4, C), which was consistent with the results of LDH release in HSEC ALI systems. Similar results were obtained after transfecting with RV-B72 RNA compared to RNA from RV-A36, C2, or C41 (data not shown).

Effects of RV species on induction of cytokines

Next we examined effects of RV species on induction of cytokine secretion, including chemokines (CCL5, CXCL8, CXCL10, and CXCL11), interferons (IFN- α 2, IFN- β , and IFN- λ 1), and IL-6, into basal medium. In general, RV infection was a potent inducer of CXCL10, CXCL11, CCL5, and IFN- λ 1. However, RV-B types induced significantly less cytokine secretion than RV-A or C at 48 hours PI and 72 hours PI (Fig 5 and Fig E6). This effect persisted even after adjusting for differences in virus load (Fig 6 and data not shown). RV also induced low level secretion of IL-6 and CXCL8, and RV-A and C types induced greater secretion of these cytokines compared to RV-B (data not shown). RV did not induce epithelial cells to secrete IFN- α 2, and induction of IFN- β was minimal (data not shown).

Effects of RV species on viral replication in PBECs

To test effects of RV species on viral replication in a different source of airway cells, we infected well-differentiated cultures of PBECs with different RV species. The species-specific effects were similar; RV-B replicated at a lower rate as compared to RV-A or RV-C (see Fig E7 in this article's Online Repository).

DISCUSSION

In this study, using a HSEC ALI system, we found that RV-B types had lower viral binding, and replicated more slowly and to lower levels as compared to RV-A and RV-C. Transfection experiments confirmed lower rates of RV-B replication in a system that bypassed receptor binding. Further, RV-B types caused less cell damage than other RV species, and induced less pro-inflammatory cytokines, chemokines, and interferons. Similar results were obtained with PBECs instead of HSECs. These findings support the hypothesis that RV-B types have reduced replication and induce a lesser cytokine response compared to RV-A or RV-C, and these characteristics are likely to contribute to the reduced virulence of RV-B that has been observed in clinical studies.^{16–20}

In this study, we used several strategies to develop an *in vitro* system that closely mimicked natural infections. First, we used clinical isolates of RVs instead of “laboratory” strains that have been propagated in cell lines over extended periods of time. It is well known that characteristics of laboratory strains of RVs can be quite different from that of clinical isolates.³⁰ The individual RV strains were selected from the phylogenetic tree to broadly represent each species.¹⁶ Seven of the nine RV types were derived from cloned cDNA, which allowed the viruses to be produced using the same protocol and cell lines. In addition, use of cloned viruses minimizes the genetic drift that can occur after repeated culturing.³¹ The error rate of picornavirus RNA polymerases has been estimated to range between 10^{-3} and 10^{-4} errors/nucleotide/cycle of replication.^{32, 33} Since plasmid DNA polymerases are much less error prone, the plasmids provide a stable source of RV sequence and allow for the production of multiple batches of viruses with genomes that are nearly identical to the original clinical isolate. Furthermore, we used fully-differentiated bronchial or sinus epithelial cells to more closely mimic conditions in the airway. This combination of viruses and cells appears to be a good model for clinical RV infection, in that the observed rate of increase of RV-A replication *in vitro* (1.5–2 log increase at 24 hours PI) is quite similar to

the rate of increase in RV RNA in nasal secretions in the first 2 days after experimental infection of volunteers with RV-A16.³⁴

As for virus quantitation, we measured virus inoculum and replication using qPCR primers that were designed to amplify each of the viral sequences with high affinity and similar efficiency. RNase A treatment was performed before RNA extraction and RT-qPCR so that the measurement would represent only RNA packaged into capsids (i.e. virions). PCR was selected for virus quantitation rather than cell culture-based techniques such as plaque assay or measuring tissue culture infective doses (TCID)₅₀/ml for several reasons. First, cell-based quantitative techniques are not yet available for RV-C. This virus does not grow in standard cell culture such as Hela cells, and it does not cause notable cytopathic effects in HSECs or PBECs at ALI. Further, for a single type, previous studies have demonstrated excellent correlation between RV measured by plaque assay or TCID₅₀/ml and that by qPCR.^{34, 35} Moreover, accurately quantitating several different RV types using cell-based techniques can be problematic since the results depend on the cell line that is used, and specific strains can require different cell lines for titering.³⁶

In a previous study, Wark et al examined the diversity in the PBEC response to infection with different RV strains.³⁰ They infected undifferentiated cell monolayers with laboratory strains of RV-A and B as well as clinical isolates, and found that infection with minor group RVs resulted in greater cytotoxicity and release of CXCR10, IL-6, and IFN- β , and less viral replication. These findings are likely related to the fact that monolayers of epithelial cells cultured in serum-free medium have ~10-fold greater expression of minor group versus major group RV receptors, leading to correspondingly higher rates of infected cells.^{37, 38}

Despite higher infection rates in monolayers *in vitro*, minor-group viruses have not been reported to cause more severe illnesses in clinical studies.^{11–21} In the Wark et al paper, viral replication, cytokine/chemokine production, and apoptosis were similar between a single clinical isolate of RV-A (A43) and that of RV-B (B48).³⁰ Differences in our findings versus those of Wark et al could be due to a number of factors related to experimental design, including differences in cell types and cultures (HSEC ALI versus undifferentiated PBEC), virus quantitation (qPCR versus cell culture), measurement of virus in cells versus media, and testing of a larger number of clinical isolates.

Several factors could contribute to reduced viral replication of RV-B types. First, viral binding of RV-B types in our experiments was lower than that of RV-A or RV-C. Since we selected only RV-A and RV-B types that bind to ICAM-1, differences between RV-A and RV-B in viral binding are independent of receptor expression levels. There are species-specific differences in the VP1 capsid sequences⁶ which may account for distinct binding properties.

In addition to reduced binding, RV-B replication after both infection and RNA transfection was slower, suggesting differences in later stages of virus life cycle such as viral protease activity or viral protein translation and RNA replication. RV encode two proteases, 2A^{pro} and 3C^{pro}, and the former interferes with host cell defenses by cleaving translation factor eIF4G to shut off cellular translation, and several nuclear pore proteins to block nucleo-

cytoplasmic trafficking. Watters et al demonstrated species-related differences in protease functions; 2A^{pro} of RV-B cleaved eIF4G or Nup62 about 4 times more slowly as compared to that of RV-A or C.³⁹ Therefore, RV-B may be less efficient at shutting off host cell antiviral responses, and the net result could be slower viral replication.

There is a positive, albeit loose, relationship between RV virus load and clinical symptoms,^{40, 41} and virus-induced cytokines, chemokines, and interferons may also contribute to respiratory symptoms.^{42–45} We demonstrated that RV-B types induce lower quantities of pro-inflammatory cytokines, which also could lead to milder illness. For example, CXCL10 may be increased in virus-induced asthma exacerbations compared to milder illnesses,^{42–44} and increased levels of CXCL10 correlate with clinical disease severity including severe airflow obstruction.⁴³ Concordant with lower rates of replication, RV-B also induced less IFN- λ 1 than RV-A or RV-C (Fig 5, J–L). Interferons have anti-viral properties,^{46, 47} but may also contribute to signs and symptoms of illness.⁴⁸ RV-B also caused less cell lyses, as indicated by lower LDH release following infection. LDH release was used as a surrogate for cell lysis, since in primary cells only a small minority of cells are infected,³⁸ and cytopathic effects are generally not evident in ALI cultures. Apoptosis can also control viral replication,^{49, 50} but we found no evidence that RV species affected apoptosis.

One limitation that should be considered in interpreting the findings of our study is that the receptor for RV-C is distinct (and as yet unknown) from that of other RV. Therefore, differences in RV-C receptor expression could complicate comparisons of binding and replication with other RV. Notably, RV-C and RV-B replicated at different rates even when cells were transfected with viral RNA to bypass cell surface receptors. Furthermore, clinical studies suggest that RV-C may have greater virulence than RV-A or RV-B.^{11–15} We did not find significant differences in epithelial cell responses to RV-C vs. RV-A, suggesting that other mechanisms, perhaps involving cells other than epithelial cells, may be important to consider in future studies.

In conclusion, RV-B types exhibit lower and slower replication and less cytopathic effects compared to RV-A or RV-C *in vitro*. RV-B induced less cytokines and chemokines such as CXCL10 in differentiated HSECs. These characteristics may contribute to reduced severity of illnesses that has been observed with infections with RV-B. Identifying the mechanisms of reduced virulence for RV-B types could lead to the development of novel therapeutic strategies for more virulent RV species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Tressa Pappas, Amy Dresen, and Fue Vang for excellent technical assistance.

Supported by the National Institute of Health Grant Nos. U19 AI104317 and P01 HL070831, and by the Banyu Fellowship Program sponsored by Banyu Life Science Foundation International (K.N.).

Disclosure of potential conflict of interest:

K. Nakagome has received research support from the National Institutes of Health (NIH) and Banyu Life Science Foundation International. Y. A. Bochkov, S. Ashraf, R. A Brockman-Schneider, M. D. Evans, and T. R. Pasic have received research support from the NIH. J. E. Gern has received research support from the NIH, Merck, AstraZeneca, and GlaxoSmithKline and received consultancy fees from Merck, GlaxoSmithKline, Biota, Centocor, Boehringer Ingelheim, MedImmune, Theraclone, and Gilead.

Abbreviations

ALI	air-liquid interface
HSEC	human sinus epithelial cell
LDH	lactate dehydrogenase
PBEC	primary bronchial epithelial cell
PI	post infection
qRT-PCR	quantitative RT-PCR
RV	rhinovirus
RV-A	RV species A
TCID	tissue culture infective doses
TER	transepithelial resistance

REFERENCES

1. Osur SL. Viral respiratory infections in association with asthma and sinusitis: a review. *Ann Allergy Asthma Immunol.* 2002; 89:553–560. [PubMed: 12487219]
2. Johnson SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, et al. Community study of role of viral infections in exacerbations of asthma in 9–11 year old children. *BMJ.* 1995; 310:1225–1229. [PubMed: 7767192]
3. Nicholson KG, Kent J, Ireland DC. Respiratory Viruses and exacerbations of asthma in adults. *BMJ.* 1993; 307:982–986. [PubMed: 8241910]
4. Friedlander SL, Busse WW. The role of rhinovirus in asthma exacerbations. *J Allergy Clin Immunol.* 2005; 116:267–273. [PubMed: 16083778]
5. Johnston SL, Pattemore PK, Sanderson G, Smith S, Campbell MJ, Josephs LK, et al. The relationship between upper respiratory infections and hospital admissions for asthma: a time-trend analysis. *Am J Respir Crit Care Med.* 1996; 154:654–660. [PubMed: 8810601]
6. Palmenberg AC, Spiro D, Kuzmickas R, Wang S, Djikeng A, Rathe JA, et al. Sequencing and analyses of all known human rhinovirus genomes reveal structure and evolution. *Science.* 2009; 324:55–59. [PubMed: 19213880]
7. Palmenberg AC, Rathe JA, Liggett SB. Analysis of the complete genome sequences of human rhinovirus. *J Allergy Clin Immunol.* 2010; 125:1190–1199. [PubMed: 20471068]
8. Gern JE. The ABCs of rhinoviruses, wheezing, and asthma. *J Virol.* 2010; 84:7418–7426. [PubMed: 20375160]
9. Bochkov YA, Gern JE. Clinical and molecular features of human rhinovirus C. *Microbes Infect.* 2012; 14:485–494. [PubMed: 22285901]
10. Kistler AL, Webster DR, Rouskin S, Magrini V, Credle JJ, Schnurr DP, et al. Genome-wide diversity and selective pressure in the human rhinovirus. *Virol J.* 2007; 4:40. [PubMed: 17477878]
11. Bizzintino J, Lee WM, Laing IA, Vang F, Pappas T, Zhang G, et al. Association between human rhinovirus C and severity of acute asthma in children. *Eur Respir J.* 2011; 37:1037–1042. [PubMed: 20693244]

12. Miller EK, Khuri-Bulos N, Williams JV, Shehabi AA, Faouri S, Al Jundi I, et al. Human rhinovirus C associated with wheezing in hospitalised children in the Middle East. *J Clin Virol.* 2009; 46:85–89. [PubMed: 19581125]
13. Miller EK, Edwards KM, Weinberg GA, Iwane MK, Griffin MR, Hall CB, et al. A novel group of rhinoviruses is associated with asthma hospitalizations. *J Allergy Clin Immunol.* 2009; 123:98–104. [PubMed: 19027147]
14. Mak RK, Tse LY, Lam WY, Wong GW, Chan PK, Leung TF. Clinical spectrum of human rhinovirus infections in hospitalized Hong Kong children. *Pediatr Infect Dis J.* 2011; 30:749–753. [PubMed: 21494174]
15. Linsuwanon P, Payungporn S, Samransamruajkit R, Posuwan N, Makkoch J, Theanboonlers A, et al. High prevalence of human rhinovirus C infection in Thai children with acute lower respiratory tract disease. *J Infect.* 2009; 59:115–121. [PubMed: 19556008]
16. Lee WM, Lemanske RF Jr, Evans MD, Vang F, Pappas T, Gangnon R, et al. Human rhinovirus species and season of infection determine illness severity. *Am J Respir Crit Care Med.* 2012; 186:886–891. [PubMed: 22923659]
17. Iwane MK, Prill MM, Lu X, Miller EK, Edwards KM, Hall CB, et al. Human rhinovirus species associated with hospitalizations for acute respiratory illness in young US children. *J Infect Dis.* 2011; 204:1702–1710. [PubMed: 22013207]
18. Fry AM, Lu X, Olsen SJ, Chittaganpitch M, Sawatwong P, Chantira S, et al. Human rhinovirus infections in rural Thailand: epidemiological evidence for rhinovirus as both pathogen and bystander. *PLoS One.* 2011; 6:e17780. [PubMed: 21479259]
19. Khetsuriani N, Lu X, Teague WG, Kazerouni N, Anderson LJ, Erdman DD. Novel human rhinoviruses and exacerbation of asthma in children. *Emerg Infect Dis.* 2008; 14:1793–1796. [PubMed: 18976575]
20. Arden KE, Chang AB, Lambert SB, Nissen MD, Sloots TP, Mackay IM. Newly identified respiratory viruses in children with asthma exacerbation not requiring admission hospital. *J Med Virol.* 2010; 82:1458–1461. [PubMed: 20572080]
21. Calvo C, Casas I, García-García ML, Pozo F, Reyes N, Cruz N, et al. Role of rhinovirus respiratory infections in sick and healthy children in Spain. *Pediatr Infect Dis J.* 2010; 29:717–720. [PubMed: 20305581]
22. Bochkov YA, Palmenberg AC, Lee WM, Rathe JA, Amineva SP, Sun X, et al. Molecular modeling, organ culture and reverse genetics for a newly identified human rhinovirus C. *Nat Med.* 2011; 17:627–632. [PubMed: 21483405]
23. Ashraf S, Brockman-Schneider R, Bochkov YA, Pasic TR, Gern JE. Biological characteristics and propagation of human rhinovirus-C in differentiated sinus epithelial cells. *Virology.* 2013; 436:143–149. [PubMed: 23199420]
24. Hao W, Bernard K, Patel N, Ulbrandt N, Feng H, Svabek C, et al. Infection and propagation of human rhinovirus C in human airway epithelial cells. *J Virol.* 2012; 86:13524–13532. [PubMed: 23035218]
25. Fotakis G, Timbrell JA. In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett.* 2006; 160:171–177. [PubMed: 16111842]
26. Lee WM, Wang W, Rueckert RR. Complete sequence of the RNA genome of human rhinovirus 16, a clinically useful common cold virus belonging to the ICAM-1 receptor group. *Virus Genes.* 1995; 9:177–181. [PubMed: 7732663]
27. Lee WM, Monroe SS, Rueckert RR. Role of maturation cleavage in infectivity of picornaviruses: activation of an infectious. *J Virol.* 1993; 67:2110–2122. [PubMed: 8383233]
28. Bochkov YA, Hanson KM, Keles S, Brockman-Schneider RA, Jarjour NN, Gern JE. Rhinovirus-induced modulation of gene expression in bronchial epithelial cells from subjects with asthma. *Mucosal Immunol.* 2010; 3:69–80. [PubMed: 19710636]
29. Schroth MK, Grimm E, Frindt P, Galagan DM, Konno SI, Love R, et al. Rhinovirus replication causes RANTES production in primary bronchial epithelial cells. *Am J Respir Cell Mol Biol.* 1999; 20:1220–1228. [PubMed: 10340941]

30. Wark PA, Grissell T, Davies B, See H, Gibson PG. Diversity in the bronchial epithelial cell response to infection with different rhinovirus strains. *Respirology*. 2009; 14:180–186. [PubMed: 19207121]
31. Cordey S, Junier T, Gerlach D, Gobbini F, Farinelli L, Zdobnov EM, et al. Rhinovirus genome evolution during experimental human infection. *PLoS One*. 2010; 5:e10588. [PubMed: 20485673]
32. Drake JW. The distribution of rates of spontaneous mutation over viruses, prokaryotes, and eukaryotes. *Ann N Y Acad Sci*. 1999; 870:100–107. [PubMed: 10415476]
33. Harvala H, Simmonds P. Human parechoviruses: biology, epidemiology and clinical significance. *J Clin Virol*. 2009; 45:1–9. [PubMed: 19372062]
34. DeMore JP, Weissshaar EH, Vrtis RF, Swenson CA, Evans MD, Morin A, et al. Similar colds in subjects with allergic asthma and nonatopic subjects after inoculation with rhinovirus-16. *J Allergy Clin Immunol*. 2009; 124:245–252. [PubMed: 19596142]
35. Mosser AG, Vrtis R, Burchell L, Lee WM, Dick CR, Weissshaar E, et al. Quantitative and qualitative analysis of rhinovirus infection in bronchial tissues. *Am J Respir Crit Care*. 2005; 171:645–651.
36. Arruda E, Crump CE, Rollins BS, Ohlin A, Hayden FG. Comparative susceptibilities of human embryonic fibroblasts and HeLa cells for isolation of human rhinoviruses. *J Clin Microbiol*. 1996; 34:1277–1279. [PubMed: 8727918]
37. Konno S, Grindle KA, Lee WM, Schroth MK, Mosser AG, Brockman-Schneider RA, et al. Interferon- γ enhances rhinovirus-induced RANTES secretion by airway epithelial cells. *Am J Respir Cell Mol Biol*. 2002; 26:594–601. [PubMed: 11970912]
38. Mosser AG, Brockman-Schneider R, Amineva S, Burchell L, Sedgwick JB, Busse WW, et al. Similar frequency of rhinovirus-infectible cells in upper and lower airway epithelium. *J Infect Dis*. 2002; 185:734–743. [PubMed: 11920291]
39. Watters K, Palmenberg AC. Differential processing of nuclear pore complex proteins by rhinovirus 2A proteases from different species and serotypes. *J Virol*. 2011; 85:10874–10883. [PubMed: 21835805]
40. Gerna G, Piralla A, Rovida F, Rognoni V, Marchi A, Locatelli F, et al. Correlation of rhinovirus load in the respiratory tract and clinical symptoms in hospitalized immunocompetent and immunocompromised patients. *J Med Virol*. 2009; 81:1498–1507. [PubMed: 19551831]
41. Takeyama A, Hashimoto K, Sato M, Sato T, Kanno S, Takano K, et al. Rhinovirus load and disease severity in children with lower respiratory tract infections. *J Med Virol*. 2012; 84:1135–1142. [PubMed: 22585733]
42. Spurrell JC, Wiehler S, Zaheer RS, Sanders SP, Proud D. Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection. *Am J Physiol Lung Cell Mol Physiol*. 2005; 289:L85–L95. [PubMed: 15764644]
43. Wark PA, Bucchieri F, Johnston SL, Gibson PG, Hamilton L, Mimica J, et al. IFN- γ -induced protein 10 is novel biomarker of rhinovirus induced asthma exacerbation. *J Allergy Clin Immunol*. 2007; 120:586–593. [PubMed: 17628646]
44. Papadopoulos NG, Xepapadaki P, Mallia P, Brusselle G, Watelet JB, Xatzipsalti M, et al. Mechanisms of virus-induced asthma exacerbations: state-of-the-art. A GA2LEN and InterAirways document. *Allergy*. 2007; 62:457–470. [PubMed: 17324199]
45. Miller EK, Hernandez JZ, Wimmenauer V, Shepherd BE, Hijano D, Libster R, et al. A mechanistic role for type III IFN- γ 1 in asthma exacerbations mediated by human rhinoviruses. *Am J Respir Crit Care Med*. 2012; 185:508–516. [PubMed: 22135341]
46. Gaajetaan GR, Geelen TH, Vernooij JH, Dentener MA, Reynaert NL, Rohde GG, et al. Interferon β induces a long-lasting antiviral state in human respiratory epithelial cells. *J Infect*. 2013; 66:163–169. [PubMed: 23201152]
47. Donnelly RP, Kotenko SV. Interferon-lambda: a new addition to an old family. *J Interferon Cytokine Res*. 2010; 30:555–564. [PubMed: 20712453]
48. Gern JE. Interferon- γ 1 and viral wheeze in asthma: a Gothic duality? *Am J Respir Crit Care Med*. 2012; 185:468–470. [PubMed: 22383563]

49. Wark PA, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V, et al. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J Exp Med*. 2005; 201:937–947. [PubMed: 15781584]
50. Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW, et al. Role of deficient type III interferon- γ production in asthma exacerbations. *Nat Med*. 2006; 12:1023–1026. [PubMed: 16906156]

Fig. 1.

Effect of RV species on viral replication in differentiated HSECs. The amount of RV RNA in adherent cells was measured just after infection (**A**) or at 24 hours PI (**B**) ($n = 5$). **C** and **D**, Viral types were combined by species. The amounts of RV RNA (**C**) and net increase in RV RNA (**D**) are shown ($n = 15$). **E**, Growth kinetics of infection of RV-A, B, and C ($n = 15$). $**P < .01$ and $***P < .001$ (versus RV-B).

Fig. 2.

Effect of inoculation dose of RVs on viral replication. The amount of RV RNA was measured just after infection (**A**) or at 24 hours PI (**B**) ($n = 5$). **C**, The relationship between the amount of RV RNA just after infection (viral binding) and that at 24 hours PI is shown. **D**, Z-score of amounts of RV RNA at 24 hours PI after adjusting for viral binding. * $P < .05$, ** $P < .01$, and *** $P < .001$ (versus RV-B52).

Fig. 3.

Effect of RV species on cellular cytotoxicity or apoptosis. **A**, Time course of cellular cytotoxicity ($n = 15$). Cellular cytotoxicity at 72 hours PI caused by individual RV types (**B**) ($n = 5$) or from groups that were combined by species (**C**) ($n = 15$). **D**, Cells were collected at 24 hours PI and caspase 3/7 activity was assessed ($n = 5$). Staurosporine (Stau; 50 μ M) was used as a positive control. $**P < .01$ (versus RV-B), $###P < .001$ (versus mock-infected cells).

Fig. 4.

Effect of RV species on viral replication and cellular cytotoxicity in WisL cells transfected with RV RNA. We transfected WisL cells with RNA of RV-A16, B52, or C15 (0.25 **(A)** or 2.5 µg **(B)**). Cells were collected and RV-RNA was measured ($n = 5$). $**P < .01$ and $***P < .001$ (versus RV-B52). **C**, Cytopathic effects observed 16 hours after transfection. Scale bars, 100 µm.

Fig. 5. Effect of RV species on cytokine production. CXCL10 (A–C), CXCL11 (D–F), CCL5 (G–I), and IFN-λ1 (J–L) concentrations were measured in basal medium after RV inoculation. Time course of cytokine production (A, D, G, and J) (n = 15). Cytokine production at 48 hours PI from individual RV types (B, E, H, and K) (n = 5) or from groups that were combined by species (C, F, I, and L) (n = 15). * $P < .05$, ** $P < .01$, and *** $P < .001$ (versus RV-B).

Fig. 6. Relationship between cytokine production and virus load. **A**, The relationship between CXCL10 concentrations and virus load at 48 hours PI. **B**, Z-score of CXCL10 concentrations after adjusting for virus load at 48 hours PI. **C**, The relationship between IFN-λ1 concentrations and virus load at 48 hours PI. **D**, Z-score of IFN-λ1 concentrations after adjusting for virus load at 48 hours PI. * $P < .05$ and *** $P < .001$ (versus RV-B).