Lower Airway Rhinovirus Burden and the Seasonal Risk of Asthma Exacerbation

Loren C. Denlinger¹, Ron L. Sorkness^{1,2,4}, Wai-Ming Lee⁴, Michael D. Evans³, Michele J. Wolff¹, Sameer K. Mathur^{1,5}, Gina M. Crisafi¹, Katie L. Gaworski¹, Tressa E. Pappas⁴, Rose F. Vrtis¹, Elizabeth A. Kelly¹, James E. Gern^{1,4}, and Nizar N. Jarjour¹

¹Departments of Medicine-Allergy, Pulmonary & Critical Care, ²Pharmacy, ³Biostatistics and Medical Informatics, and ⁴Pediatrics-Allergy & Immunology, University of Wisconsin-Madison, Madison, Wisconsin, ⁵William S. Middleton VA Hospital, Madison, Wisconsin

Rationale: Most asthma exacerbations are initiated by viral upper respiratory illnesses. It is unclear whether human rhinovirus (HRV)-induced exacerbations are associated with greater viral replication and neutrophilic inflammation compared with HRV colds.

Objectives: To evaluate viral strain and load in a prospective asthma cohort during a natural cold.

Methods: Adults were enrolled at the first sign of a cold, with daily monitoring of symptoms, medication use, and peak expiratory flow rate until resolution. Serial nasal lavage and induced sputum samples were assessed for viral copy number and inflammatory cell counts.

Measurements and Main Results: A total of 52 persons with asthma and 14 control subjects without atopy or asthma were studied for over 10 weeks per subject on average; 25 participants developed an asthma exacerbation. Detection of HRVs in the preceding 5 days was the most common attributable exposure related to exacerbation. Compared with other infections, those by a minor group A HRV were 4.4-fold more likely to cause exacerbation ($P = 0.038$). Overall, sputum neutrophils and the burden of rhinovirus in the lower airway were similar in control subjects without atopy and the asthma group. However, among HRV-infected participants with asthma, exacerbations were associated with greater sputum neutrophil counts ($P = 0.005$).

Conclusions: HRV infection is a frequent cause of exacerbations in adults with asthma and a cold, and there may be group-specific differences in severity of these events. The absence of large differences in viral burden among groups suggests differential lower airway sensitization to the effects of neutrophilic inflammation in the patients having exacerbations.

Keywords: asthma; virus; exacerbation; index; neutrophil

Acute asthma exacerbations remain a significant cause of morbidity and accelerated decline in lung function (1–3). Although symptomatic upper respiratory tract viral illnesses are the dominant risk factor in pediatric exacerbations, this link is less well established in adults (4). One prospective cohort of 138 adult

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

There is continued controversy with respect to the contribution of viral upper respiratory tract infections to asthma exacerbations in adults, and whether viral strains differ in terms of the associated lower airway burden and likelihood of causing exacerbation.

What This Study Adds to the Field

Exposure to minor group A human rhinoviruses (HRVs) had the highest rates of exacerbations in this study. In patients with asthma, those with an exacerbation have higher sputum neutrophil counts than those with a routine cold, without large differences in HRV burden between groups.

patients with asthma recruited at baseline and followed for over 1 year showed that 71% of adult exacerbations were preceded by upper respiratory symptoms (5). Adults with asthma experience the same number of colds as their domestic partners without asthma, but are twice as likely to develop lower airway symptoms (6). Numerous studies with enrollment at the time of exacerbation show association with respiratory viruses at a wide range of detection (26–78%) (5, 7–14). The timing of sample acquisition in a natural infection may be critical, given that the intensity of cold symptoms in the first 2 days is more predictive of worsening asthma control than cold scores reported by participants after the second day (15). In short, these challenges contribute to an incomplete understanding of the pathophysiology of asthma exacerbations in adults, and potentially hinder development of more effective treatments to be used specifically when asthma control is threatened (16).

Although virus-induced asthma exacerbations are most frequently associated with rhinoviruses, not all colds result in exacerbations, highlighting interaction between the pathogen and host. Inoculation studies with laboratory strains of human rhinovirus (HRV) have demonstrated significant variability in the host immune responses to HRV infections (17–23), although the magnitude and timing of this response in relation to the overall inflammatory profile is not resolved (24–26). Neutrophilic inflammation of the lower airways has been demonstrated to be a feature of exacerbations (27, 28); however, less attention has been paid to the molecular virology of natural colds contributing to acute asthma. Sequence analysis demonstrates there are over 150 HRV strains (29). Major HRVs bind to intercellular adhesion molecule (ICAM)-1, and include most of serogroup A and all of group B (30–32). Minor HRV is a small subset of serogroup A that bind to the low-density lipoprotein receptor (LDL-R) (33). HRV-C has been described more recently and does not appear to bind to either ICAM-1 or LDL-R. There is

(Received in original form March 31, 2011; accepted in final form August 4, 2011)

Supported by National Heart, Lung, and Blood Institute (NHLBI) grant R01 HL080412, National Institutes of Health (NIH) NHLBI grant K23 HL081492, NIH National Institute of Allergy and Infectious Diseases grant U19 AI070503, and NIH National Center for Research Resources grant 1 UL1RR025011.

Author contribution: conception, design, and data collection—all authors; analysis and interpretation—L.C.D., R.L.S., W.-M.L., M.D.E., S.K.M., E.A.K., J.E.G., and N.N.J.; drafting the manuscript for important intellectual content—L.C.D., R.L.S., W.-M.L., M.D.E., S.K.M., E.A.K., J.E.G., and N.N.J.

Correspondence and requests for reprints should be addressed to Loren C. Denlinger, M.D., Ph.D., Division of Allergy, Pulmonary & Critical Care Medicine, University of Wisconsin School of Medicine & Public Health, 600 Highland Avenue, CSC Box 9988, Madison, WI 53792. E-mail: lcd@medicine.wisc.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 184, pp 1007–1014, 2011

Originally Published in Press as DOI: 10.1164/rccm.201103-0585OC on August 4, 2011
Internet address: www.atsjournals.org

suggestion that the groups differ in pathogenicity, in that HRV-C infections were more commonly found in hospitalized children than other strains (34–36). Moreover, several studies have shown that the lower airway can be infected by rhinovirus, but whether this potential exists differentially by strain has not been evaluated (37–40). As such, the objective of this study was to determine the relationships between acute asthma symptoms, HRV group, and lower airway viral burden in the setting of natural infections.

We hypothesized that the rate of asthma exacerbation differs among HRV group and that lower airway viral burden driving an acute neutrophilic inflammatory response correlates with acute asthma symptoms and the risk of exacerbation. Herein, we describe a prospective cohort of patients with asthma and healthy volunteers recruited during cold seasons and enrolled at the first onset of a naturally occurring upper respiratory infection (URI) before asthma symptoms developed. Two poster abstracts discussing a portion of these results have been previously published (41, 42).

METHODS

Study Design and Exacerbation Definition

The study was approved by the University of Wisconsin Health Sciences Institutional Review Board. All participants provided written, informed consent, with recruitment during the fall and spring cold seasons from 2005 through 2009. Enrollment required a modified Jackson cold symptom score of at least 2, as previously described (21, 43, 44). Figure 1 shows the basic study design. All subjects completed eight visits, including seven opportunities to collect nasal lavage samples and two induced sputa. The first 4 visits (acute) occurred within the first 14 days from the onset of the cold. Visits 5 and 6 during the recovery period were primarily for nasal lavage collection and diary validation. To estimate the precold baseline state, the last two visits (resolution) were not scheduled until all upper and lower respiratory symptoms were resolved. Details regarding the participant entry criteria and the following procedures are described in the online supplement: skin testing; aeroallergen counting; lung function measurement; nasal lavage; induced sputum collection; sample processing; and assessment of exhaled nitric oxide.

Exacerbations were defined as an asthma index score greater than 30, as previously described (with an online-available worksheet [45]). The time to the first exacerbation was also recorded for all events. Additional details are presented in the online supplement.

Virus Detection, Rhinovirus Molecular Typing, and Quantification

Nasal lavage and sputum RNA samples were extracted from aliquots of the cell pellet containing 500,000 cells after storage in Trizol Reagent (Invitrogen, Carlsbad, CA). After reverse transcription to cDNA by standard methods, the Respiratory Multicode Assay (Eragen Biosciences, Madison, WI) was used for fluorimetric detection of the presence of common respiratory viruses with multiplex-polymerase chain reaction (PCR) and flow cytometry (46). Samples positive for

rhinoviruses were partially sequenced (260-bp variable region in the 5' noncoding genome) to determine strain (34). Quantitative PCR was then performed with a universal rhinovirus primer set using an ABI Prism 7,000 machine (Applied Biosystems, Carlsbad, CA). The oligonucleotide sequences for these primers are as follows: PCR forward primer, CTAGCCTGCGTGG (D110); PCR reverse primer, AAACA CGGACACCCAAAGTAGT (RVQ1); and probe, 6FAM-TCCTC CGGCCCCCTGA-MGB-NFQ (RVQ2). These primers correspond to a conserved region of the 5' untranslated region, and were validated against 97 HRV strains, with further refinement after consideration of sequences from field strains, including HRV-C. As an estimate of viral burden similar to methods described previously (47), HRV copy number was determined by extrapolation of real-time PCR data to a standard curve generated from a stock of HRV16 with known concentration in terms of plaque-forming units.

We employed the following definitions: (1) viral infections referred to the presence of the same virus in at least two contiguously collected samples; (2) viral illnesses were the infections occurring in conjunction with upper respiratory symptoms greater than the 95% confidence interval of Jackson symptom scores reported at the resolution visits; (3) virus-associated exacerbations were when the first detection of a given virus strain occurred within 5 days before the exacerbation, or up to 2 days after the start of the exacerbation if no antecedent respiratory sample had been collected.

Statistical Analyses

Based on data from our previous studies, and assuming a 40% exacerbation rate and a threefold difference between groups in the acute-to-baseline change of sputum neutrophils, we calculated that 51 patients with asthma would provide 80% power to test the hypothesis that URI-preceded asthma exacerbations are associated with increased sputum neutrophils compared with patients with asthma and URIs who do not exacerbate. The primary analysis plan also included comparison of the hazard rates of exacerbation for infection by different HRV groups, as well as comparisons of HRV copy number between groups of participants with asthma stratified by exacerbation status. Secondary endpoints included the development of a logistic regression model describing acute and postcold resolution factors predicting exacerbation status during the first four visits (see the online supplement for details), as well as comparisons of virus copy number and neutrophil count between all of the participants with asthma collectively to those values obtained from the nonatopic, nonasthmatic control group. Variables not falling under normal distributions were adjusted with log, natural log, or square root transformations, as appropriate. Statistical analysis and graphing were performed with JMP 8 (SAS, Cary, NC) and SigmaPlot 11 (SysStat Software, San Jose, CA). Testing methods for all comparisons are described in the text, and a *P* value less than 0.05 was considered significant.

RESULTS

Participant, Cold, and Exacerbation Characteristics

A total of 134 volunteers, including 84 with asthma, were recruited during 9 cold seasons, involving a total of 803 person-weeks of

Figure 1. Study visit structure and procedures. Participants were recruited to enroll in the fall and spring seasons by mass e-mail, radio, and newspaper advertisements with instructions to present within 48 hours of the onset of cold symptoms. The first 4 visits were completed within 14 days of the onset of cold symptoms, with the vast majority of the first 4 visits completed within 7 days

of onset. The first resolution visit was scheduled only after cold and acute lower airway symptoms were no longer present. Seven nasal lavage (NL) and two induced sputum samples were collected as shown. Similarly, spirometry, exhaled nitric oxide (FeNO), and methacholine challenge tests were performed as shown.

follow-up, and a total of 768 respiratory samples processed for virus detection. Historical data showed that, of the subjects with asthma, 28 had used prednisone, 35 required an emergency department visit, 11 were hospitalized, and 2 required intensive care for asthma in the past. A total of 71 subjects completed all of the visits. One subject was excluded due to restrictive lung physiology, and four were not analyzed because of incomplete diary data, precluding calculation of the asthma index. Of the remaining 66 participants, 52 had allergic asthma and 14 were nonatopic and did not have asthma (Table 1). The distributions of age and sex did not differ by study completion status (ANOVA $P = 0.751$, Chi-squared $P = 0.138$, respectively). Unfortunately, and despite similar illness severity, non-White participants were 2.5 times more likely to withdraw prematurely from the study, such that the resulting study population is 94% White (Chi-squared $P = 0.002$). Nonetheless, the incidence of prior prednisone bursts or advanced medical care did not differ between the asthma participants completing or withdrawing from this study (23 of 52 completing, 12 of 31 withdrawing; Chi-squared $P = 0.622$).

All participants had cold symptom scores on the day of enrollment (13.6 ± 5.5) and peak acute scores (15.5 ± 5.3) that were higher than the individual baseline averages (1.1 ± 2.1 ; $P < 0.001$ for both comparisons). Rates of viral infection were high ($\geq 89\%$) and did not differ among subject groups (Table 1). A total of 39 of the 52 (75%) patients with asthma and 11 of the 14 (79%) control subjects were infected with rhinovirus during the study, and the distribution of rhinovirus serotype groups is shown in Table 2. Other viruses detected in 10 patients with asthma and 3 control subjects included coronavirus ($n = 3$), metapneumovirus ($n = 2$), enterovirus ($n = 2$), influenza A ($n = 3$), influenza B ($n = 1$), parainfluenza-2 ($n = 1$), and respiratory syncytial virus B ($n = 1$). A total of 45 of the 55 participants with viruses detected in nasal lavage samples collected at the first two visits also had a virus present in the sputum sample, with matching strains in all cases comparing the upper and lower respiratory isolates. A total of 16 participants had more than one infection, which usually were separated by a period of health in the absence of cold symptoms. Only one subject had a mixed infection with more than one virus strain (respiratory syncytial virus-b and coronavirus), but this did not result in an exacerbation. Finally, there were five asymptomatic infections that occurred after cold symptoms had resolved, with HRV group distributions shown in Table 2.

A total of 29 exacerbations occurred during the course of the study in 25 subjects, with a geometric average time to the first

exacerbation of 7 ± 2 days from the onset of the cold. Figure 2A shows the cold symptom scores, asthma index, and the timing of respiratory sampling in a representative patient, whereas Figure 2B shows the profile of a subject whose initial cold symptoms occurred in the absence of virus detection. A total of 19 of the 25 first exacerbations were associated with HRV, in that the first detection of virus occurred within 5 days before the exacerbation or up to 2 days after the event if no antecedent sample existed. HRV-associated exacerbations occurred on a geometric mean of 6 ± 2 days after the onset of cold symptoms, compared with 13 ± 3 days to manifest non-HRV-associated exacerbations (ANOVA $P = 0.048$). Comparing patients with asthma with and without exacerbations, there was no difference in lung function at the first visit, prior use of inhaled corticosteroid (ICS), or number of positive skin tests (Table 1). Despite characteristics at study entry similar to nonexacerbators, participants with an asthma exacerbation at any time during the study had more airflow obstruction and greater asthma index variability in the resolution period (see Table E1 in the online supplement).

Minor Group A Rhinovirus as the Primary Seasonal Exposure Associated with Exacerbations

Qualitative detection of any rhinovirus was the largest risk factor for exacerbation in a logistic regression model (Table E2), and this finding remained significant after adjustment for pre-enrollment ICS use and FEV₁ measured at resolution (adjusted OR, 6.8; 95% confidence interval, 1.6–30.0; $P = 0.01$). With respect to HRV group differences, seven of nine subjects exposed to a minor group A rhinovirus had an exacerbation, which was the highest proportion of subjects at risk for their first event (Table 2). Hazard rates for each HRV group were apportioned using the follow-up time after the first virus detection. Compared with all other viral groups and environmental exposures combined, that hazard ratio of having an exacerbation after minor group A rhinovirus exposure was 4.4 ($P = 0.038$; Table 2).

Lower Airway Rhinovirus and Neutrophil Burden Are Associated with Asthma Exacerbations

The peak of nasal lavage rhinovirus copy number occurred on a median of 3 days after the self-reported onset of symptoms, with an interquartile range of 2–4 days; this result is similar to findings in inoculation studies (26). The geometric mean of HRV copies in the sputum samples from patients having an exacerbation was numerically 6.4-fold higher than those not having an exacerbation, although this did not reach statistical

TABLE 1. PATIENT CHARACTERISTICS AT THE ENROLLMENT VISIT, AND RATES OF VIRUS EXPOSURE THROUGHOUT THE STUDY

	Asthma with Subsequent Exacerbation	Asthma without Exacerbation	Control Subjects without Atopy or Asthma	All Groups, <i>P</i> Value	Asthma Only, <i>P</i> Value
Participants, <i>n</i>	25	27	14		
Age, yr	29 ± 12	24 ± 6	27 ± 11	0.242	0.085
Sex, % female	76	70	57	0.468	0.648
FEV ₁ % predicted at visit 1	95 ± 14	95 ± 13	102 ± 10	0.238	0.895
FEV ₁ /FVC at visit 1	0.77 ± 0.08	0.81 ± 0.08	0.84 ± 0.08	0.036	0.151
Albuterol-FEV ₁ % change at visit 1	6 ± 7	6 ± 6	3 ± 3	0.214	0.798
Prior ICS use, <i>n</i>	8	7	0	N.D.	0.629
Virus positive, <i>n</i>	23	24	14	0.441	0.704
Positive skin tests per participant	6 ± 3	5 ± 3	0	N.D.	0.252
Follow-up, wk	12 ± 4	10 ± 3	9 ± 4	0.040	0.040

Definition of abbreviations: ICS = inhaled corticosteroid; N.D. = not determined.

All participants had acute cold symptoms upon enrollment. Those with asthma were stratified by the presence or absence of any exacerbation during the entire course of the study. Virus exposure is listed as the number of subjects in each group with any respiratory sample positive for viruses. Continuous data are represented as mean ± SD. ANOVA or Student's *t* tests were used for testing continuous data from all groups of subjects or just those with asthma, respectively. Chi-squared tests are presented for categorical data.

TABLE 2. RATES OF INFECTION, UPPER RESPIRATORY ILLNESS, AND ASTHMA EXACERBATIONS BY DETECTED GROUPS OF RHINOVIRUS

Virus Group	Viral Infections	Viral Illnesses	Subjects at Risk for First Exacerbation	Subjects with at Least One Exacerbation	Person-Weeks of Follow-Up to First Exacerbation or Censure	Hazard Rate	P Value (vs. All Others Combined)
HRV-A major	14	14	12	7	49.7	0.141	0.210
HRV-A minor	11	10	9	7	24.3	0.288	0.038
HRV-B	11	8	8	4	58.4	0.068	0.896
HRV-C	10	10	8	3	55.6	0.054	0.448
HRV—not typable	4	3	1	1	1.1	0.875	0.371
Other viruses	10	10	8	1	64.1	0.016	0.053
None detected before exacerbation	0	0	6	2	44	0.045	0.491
Total	60	55	52	25	297.2	0.084	—

Definition of abbreviation: HRV = human rhinovirus.

Viral infections in the participants with asthma at any time during the study are enumerated by group. When viruses were detected during the acute visits, nearly all were present in the first sample (give number in first sample/total viruses detected), and the median duration of viral shedding was 10 days (interquartile range, 7–19 d). A total of 35 subjects had only 1 virus detected; 11 subjects had 2 and 1 subject experienced 3 infections during the course of the study, usually representing distinct colds. Five viral infections were asymptomatic. Viral illnesses that occurred before the first exacerbation are presented with the number of exacerbations, person-weeks of follow-up, and associated hazard rates. A proportional hazards model tested the differences between individual HRV groups.

significance with the current sample size ($21.2 \pm 4.9 \times 10^5$ versus $3.3 \pm 0.7 \times 10^5$ copies; $P = 0.123$). To evaluate whether some individuals had a higher burden of HRV in the lower airway than that detected in the nose, we expressed the data as a ratio of the sputum copy number to the maximum found in the nasal lavage samples for each participant. Figure 3A

Figure 2. Timing of NL samples and the definition of virus-associated exacerbations. The daily highest total symptom score for the modified Jackson cold symptom survey is shown with filled circles, whereas the asthma index is shown with open triangles. The timing of respiratory sampling is also denoted with arrows referring to virus detection and open circles denoting virus-negative samples. (A) A subject with a typical profile is depicted, whereas (B) depicts a subject whose upper respiratory symptoms were initially not associated with virus detection, but eventually acquired a virus and had a human rhinovirus (HRV)-associated exacerbation.

demonstrates that patients having a rhinovirus-triggered asthma exacerbation are more likely than those without an exacerbation to have a sputum-to-nasal lavage HRV ratio greater than 1, suggestive of replication of the virus in the lower airway. There were no differences in viral load in the sputum samples among classes of HRV.

Subjects with asthma exacerbations of any cause had higher neutrophil counts in the sputum samples collected at the acute visit than did participants without an asthma exacerbation (Figure 3B). This was also the case when restricting the analysis to HRV-exposed individuals, with a geometric mean of sputum polymorphonuclear cells (PMNs) that was 5.5-fold higher in patients with exacerbations compared with those without ($P = 0.005$). To estimate the influx of PMNs during the cold, we also evaluated the acute-to-baseline ratio of sputum PMNs. Univariate logistic regression analysis showed that, for every twofold increase in the acute-to-baseline sputum PMN ratio, the odds ratio of exacerbation was 1.4 (95% confidence interval, 1.0–1.9; $P = 0.04$; Table E2). Finally, there were no differences in viral burden or sputum neutrophil counts between patients with asthma and the control participants without atopy (Figure E1).

Secondary Endpoints and Post Hoc Analyses

We performed logistic regression analysis on data from subjects with asthma to test for associations between subject and illness characteristics and asthma exacerbation (Table E2). The severity of colds, duration of upper respiratory symptoms, qualitative detection of rhinovirus, and neutrophil influx were all associated with exacerbations. Upon recovery from the cold, the percent predicted FEV₁/FVC ratio was the only variable that significantly related to exacerbation. Similar results were obtained in analyzing the asthma index as a continuous variable (data not shown). In addition, 33 of the 52 subjects with allergic asthma had upper respiratory symptoms that coincided with environmental exposure to relevant allergens. The rates of seasonal symptomatic allergen exposures did not differ according to exacerbation status ($P = 0.581$), nor did it change the duration of upper respiratory symptoms in subjects with asthma (14 ± 7 versus 18 ± 13 d, without and with seasonal exposure respectively; $P = 0.220$). Three subjects had exacerbations likely due to *Alternaria* sensitization and exposure in the absence of virus detection; however, the small number of patients in this group did not permit a reliable estimate of the hazard rate. Moreover, neither the change of sputum eosinophils or nor change in exhaled nitric oxide levels were predictive of exacerbations in the entire cohort (see the online supplement, including Table E2).

Figure 3. Rhinovirus and sputum neutrophil burden in acute samples. Quantitative PCR was performed using universal HRV primers on all available acute samples from all participants with a detected rhinovirus (A), stratified by the presence or absence of an exacerbation occurring within 5 days of collection of the sputum sample (which occurred on Cold Day 5 ± 2 in the acute period). The sputum HRV burden is expressed as a ratio to the peak occurring in the NL samples. Chi-squared analysis was done for the rates of having more HRV copies in the sputum sample than in the peak of the NL, with sample sizes of 13 and 14, respectively. (B) The absolute number of neutrophils per gram of sputum during the acute visit. Sputum neutrophil data from all subjects with asthma are shown regardless of virus detection ($n = 28$ and 19, respectively). Data were log transformed before ANOVA testing. All data are expressed as box and whisker plots.

Finally, participants taking ICS before enrollment had a greater change in sputum eosinophils and a trend toward a higher sputum HRV copy number compared with subjects not taking ICS (Figure E2). By contrast, the change in exhaled nitric oxide levels did not differ according to the presence or absence of ICS use before the cold (Figure E2).

DISCUSSION

The goals of this study were to determine whether rhinovirus colds were associated with distinct patterns of viral replication or neutrophilic airway inflammation in subjects with asthma, and to determine whether these same predictors correlated with exacerbations. Three-fourths of the colds in this study were due to rhinoviruses, and HRV exposure was the primary agent associated with exacerbation in the following 5 days. This rate is at

the higher end of those reported in other studies (5, 8–12), and may, in part, reflect recruitment strategies designed to enroll subjects at the first sign of a cold and before the exacerbation occurs. Data from the present study provide several novel insights. Foremost, the rate of exacerbations was 4.4-fold higher for group A minor HRV compared with all other groups combined, and may be the first study in adults with asthma to examine differences in HRV group pathogenicity. Published studies in children suggest that HRV-C may be more likely to cause severe exacerbations requiring hospitalization (34–36, 48). HRV-C is more difficult to grow in culture, and does not appear to bind ICAM-1 or the LDL-R (49). Although we did not perform broad surveillance of adults for a complete profile of HRV strains in the community for each cold season, HRV group A has been shown to be more likely to have an increased symptom burden in general populations of adults (50, 51). Collectively, these data suggest that there may be an age dependence to the intensity of the immune response to HRV, or that prior exposure to HRV-C may provide greater protection against subsequent exposure to this group in adults, in comparison with other HRV groups. Other potential mechanisms for varying HRV group pathogenicity that warrant further study include baseline differences in lower airway epithelial cell expression levels of ICAM-1, LDL-R, and the receptor for HRV-C, inconsistent effects of ICS on the expression of these receptors, and/or potential divergent signaling pathways, leading to production of chemokines and/or antiviral factors (52–54).

The quantitative assessment of HRV copy number and associated lower airway inflammation in patients with asthma relative to control subjects without atopy or asthma has also not been previously shown during natural infections. We demonstrate that the HRV copy number in acute sputum samples from patients with asthma are not different when grouped according to exacerbation status; however, patients with HRV-associated exacerbations had greater than fivefold more neutrophils in their acute sputum samples than subjects with HRV colds, but no exacerbation. These data can be interpreted in several ways. First, it is likely that the variability in measuring HRV RNA copy number in a sputum sample is greater than that associated with neutrophil enumeration. In this sense, the present study may be underpowered to detect differences in lower airway HRV copy number that would be predicted if patients with asthma indeed have a relative deficiency in antiviral factors (22, 23). Consistent with this notion is our observation that select patients with exacerbations have a sputum-to-peak nasal lavage HRV ratio greater than 1, suggestive of lower airway replication. In this light, it was interesting that *post hoc* analysis showed a trend toward increased sputum HRV copy number in patients previously taking ICS. Previous ICS use may be a marker for attenuated antiviral host factor production, and, similarly, administration of oral steroids has been shown to increase the nasal HRV titer during inoculation experiments, such that the increased burden may be an effect of the medicine (55). Second, patients with exacerbations could have more robust recruitment and/or delayed clearance of neutrophils and other inflammatory cells from the lower airway in the setting of a similar viral challenge. With respect to the latter possibility, defects in alveolar macrophage phagocytosis have been associated with severe asthma phenotypes that are more exacerbation prone (56, 57). Third, given that the control subjects who are HRV infected and nonatopic in the present study had similar sputum neutrophil counts to those from the patients with asthma, there are likely to be additional features of the asthmatic lower airway that contribute to the exacerbation phenotype. Collectively, these observations will need validation in a larger cohort, with the goal of identifying biomarkers related to the risk of exacerbations during natural infections.

Clearly, the timing of respiratory sample collection is critical in a natural infection and, as such, our study design had a number of strengths and some limitations that should be considered in interpreting these findings. To fully characterize the effects of the acute illness, all participants completed four study visits within the first 14 days after the onset of cold symptoms. That the cold symptom scores in all participants continued to rise during the early visits, the peak nasal lavage HRV copy number occurred on the third day of cold symptoms (similar to that observed in inoculation experiments (26)), and lung function at the first visit was not different between patients with asthma with or without exacerbation, all suggest that our recruiting efforts enrolled a pre-exacerbation population. Nonetheless, it is possible that some of the acute samples were collected after peak inflammation. The ability to test multiple samples of respiratory secretions with sensitive and comprehensive PCR-based viral diagnostics (58) adds to the validity of identifying viral illnesses. Furthermore, HRV-positive samples were confirmed by sequencing and molecular typing, which allowed for analysis of group-specific effects. In contrast, previous work by others identified female sex, race, peak expiratory flow variability, lack of use of ICS, and a history of treatment with oral corticosteroid, such as prednisone, as independent risk factors for the incidence and severity of asthma exacerbations (1, 2, 59). These factors were not significant in the present study, and may reflect a limitation of our sample size and mild baseline disease impairment. Of note, 36 of the initial 84 patients with asthma (42.9%) had required prednisone and/or advanced medical care during episodes before the current study, demonstrating that our recruiting strategy captured an at-risk population. Our entry criteria required an $FEV_1 \geq 70\%$ predicted at the first visit, and an absence of baseline use of high-dose ICS or prednisone to ensure safety during the complex visit structure and procedural burden. As such, our findings may not apply to those with more severe asthma, although this population will clearly be of interest in terms of the potential interactions between corticosteroids and viral burden. Use of recently adapted nasal sample collection methods at home (47) may allow us to enroll patients in future protocols with a greater spectrum of disease severity and fewer dedicated study visits.

In conclusion, we show that HRV infection is the most frequent exposure in adults causing seasonal asthma exacerbation in the setting of concomitant upper respiratory symptoms, and that minor group A HRV has the highest rate. Relative to participants with asthma experiencing an HRV cold only, subjects with exacerbations have higher sputum neutrophil counts and an increased chance of having more HRV detected in the sputum compared with the nasal samples. Ongoing work in several laboratories is focused on the identity of rhinovirus species or sequence elements that confer the most pathogenic risk. Additional study is also warranted regarding the mechanisms by which allergic inflammation influences the antiviral response and/or sensitizes the lower airway to injury associated with neutrophilic influx.

Author Disclosure: L.C.D. has received consultancy fees from Merck, Inc. (\$1,000 in 2009); his institution has received funding for two issued patents from the Wisconsin Alumni Research Foundation. R.L.S. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.-M.L. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.D.E. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.J.W. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.K.M. received consultancy fees from Cephalon, Inc.; he is employed by the University of Wisconsin, Madison. G.M.C. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.L.G. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.E.P. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.F.V. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.A.K. has no financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.E.G.

has received consultancy fees from GlaxoSmithKline (GSK), Biota, Centocor, Synairgen, 3V Biosciences, Boehringer Ingelheim, and has stock options in 3V BioSciences and EraGen Biosciences; his institution has received grants from Merck Inc. and AstraZeneca. N.N.J.'s institution has received grants from Merck, GSK, Pharmaxis, and Genetech; he has received consultancy fees from Asthmatx, Genetech, and GSK; he received an honorarium from the Saudi Thoracic Society for a GSK-supported continuing medical education program.

Acknowledgment: Above all, the authors thank the participants of this study for their dedication to asthma research. In addition, they thank Cheri Swenson, Evelyn Falbene, and Erin Billmeyer for initial help with protocol development, subject recruitment, characterization, and database forms creation. They are grateful to Dr. Shachar Peles for scoring the skin test data.

References

1. Tattersfield AE, Postma DS, Barnes PJ, Svensson K, Bauer CA, O'Byrne PM, Lofdahl CG, Pauwels RA, Ullman A. Exacerbations of asthma: a descriptive study of 425 severe exacerbations. The Facet International Study Group. *Am J Respir Crit Care Med* 1999;160:594–599.
2. Weber EJ, Silverman RA, Callahan ML, Pollack CV, Woodruff PG, Clark S, Camargo CA Jr. A prospective multicenter study of factors associated with hospital admission among adults with acute asthma. *Am J Med* 2002;113:371–378.
3. O'Byrne PM, Pedersen S, Lamm CJ, Tan WC, Busse WW. Severe exacerbations and decline in lung function in asthma. *Am J Respir Crit Care Med* 2009;179:19–24.
4. Jackson DJ, Johnston SL. The role of viruses in acute exacerbations of asthma. *J Allergy Clin Immunol* 2010;125:1178–1187; quiz 1188–1179.
5. Nicholson KG, Kent J, Ireland DC. Respiratory viruses and exacerbations of asthma in adults. *BMJ* 1993;307:982–986.
6. Corne JM, Marshall C, Smith S, Schreiber J, Sanderson G, Holgate ST, Johnston SL. Frequency, severity, and duration of rhinovirus infections in asthmatic and non-asthmatic individuals: a longitudinal cohort study. *Lancet* 2002;359:831–834.
7. van Elden LJ, Sachs AP, van Loon AM, Haarman M, van de Vijver DA, Kimman TG, Zuithoff P, Schipper PJ, Verheij TJ, Nijhuis M. Enhanced severity of virus associated lower respiratory tract disease in asthma patients may not be associated with delayed viral clearance and increased viral load in the upper respiratory tract. *J Clin Virol* 2008;41:116–121.
8. Wark PA, Johnston SL, Moric I, Simpson JL, Hensley MJ, Gibson PG. Neutrophil degranulation and cell lysis is associated with clinical severity in virus-induced asthma. *Eur Respir J* 2002;19:68–75.
9. Grissell TV, Powell H, Shafren DR, Boyle MJ, Hensley MJ, Jones PD, Whitehead BF, Gibson PG. Interleukin-10 gene expression in acute virus-induced asthma. *Am J Respir Crit Care Med* 2005;172:433–439.
10. Johnston SL, Pattemore PK, Sanderson G, Smith S, Campbell MJ, Josephs LK, Cunningham A, Robinson BS, Myint SH, Ward ME, 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.
11. Atmar RL, Guy E, Guntupalli KK, Zimmerman JL, Bandi VD, Baxter BD, Greenberg SB. Respiratory tract viral infections in inner-city asthmatic adults. *Arch Intern Med* 1998;158:2453–2459.
12. Green RM, Custovic A, Sanderson G, Hunter J, Johnston SL, Woodcock A. Synergism between allergens and viruses and risk of hospital admission with asthma: case-control study. *BMJ* 2002;324:763.
13. Jacoby DB. Virus-induced asthma attacks. *JAMA* 2002;287:755–761.
14. Guilbert TW, Denlinger LC. Role of infection in the development and exacerbation of asthma. *Expert Rev Respir Med* 2010;4:71–83.
15. Walter MJ, Castro M, Kunselman SJ, Chinchilli VM, Reno M, Ramkumar TP, Avila PC, Boushey HA, Ameredes BT, Bleecker ER, et al. Predicting worsening asthma control following the common cold. *Eur Respir J* 2008;32:1548–1554.
16. Rosenthal LA, Avila PC, Heymann PW, Martin RJ, Miller EK, Papadopoulos NG, Peebles RS Jr, Gern JE. Viral respiratory tract infections and asthma: the course ahead. *J Allergy Clin Immunol* 2010;125:1212–1217.
17. Calhoun WJ, Dick EC, Schwartz LB, Busse WW. A common cold virus, rhinovirus 16, potentiates airway inflammation after segmental

- antigen bronchoprovocation in allergic subjects. *J Clin Invest* 1994;94:2200–2208.
18. Lemanske RF Jr, Dick EC, Swenson CA, Vrtis RF, Busse WW. Rhinovirus upper respiratory infection increases airway hyperreactivity and late asthmatic reactions. *J Clin Invest* 1989;83:1–10.
 19. Grunberg K, Timmers MC, Smits HH, de Klerk EP, Dick EC, Spaan WJ, Hiemstra PS, Sterk PJ. Effect of experimental rhinovirus 16 colds on airway hyperresponsiveness to histamine and interleukin-8 in nasal lavage in asthmatic subjects *in vivo*. *Clin Exp Allergy* 1997;27:36–45.
 20. Jarjour NN, Gern JE, Kelly EA, Swenson CA, Dick CR, Busse WW. The effect of an experimental rhinovirus 16 infection on bronchial lavage neutrophils. *J Allergy Clin Immunol* 2000;105:1169–1177.
 21. Gern JE, Vrtis R, Grindle KA, Swenson C, Busse WW. Relationship of upper and lower airway cytokines to outcome of experimental rhinovirus infection. *Am J Respir Crit Care Med* 2000;162:2226–2231.
 22. Wark PA, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V, Holgate ST, Davies DE. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J Exp Med* 2005;201:937–947.
 23. Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW, Keadze T, Mallia P, Stanciu LA, Parker HL, *et al.* Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med* 2006;12:1023–1026.
 24. Fleming HE, Little FF, Schnurr D, Avila PC, Wong H, Liu J, Yagi S, Boushey HA. Rhinovirus-16 colds in healthy and in asthmatic subjects: similar changes in upper and lower airways. *Am J Respir Crit Care Med* 1999;160:100–108.
 25. Fraenkel DJ, Bardin PG, Sanderson G, Lampe F, Johnston SL, Holgate ST. Lower airways inflammation during rhinovirus colds in normal and in asthmatic subjects. *Am J Respir Crit Care Med* 1995;151:879–886.
 26. DeMore JP, Weisshaar EH, Vrtis RF, Swenson CA, Evans MD, Morin A, Hazel E, Bork JA, Kakumanu S, Sorkness R, *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.
 27. Fahy JV, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol* 1995;95:843–852.
 28. Pizzichini MM, Pizzichini E, Efthimiadis A, Chauhan AJ, Johnston SL, Hussack P, Mahony J, Dolovich J, Hargreave FE. Asthma and natural colds. Inflammatory indices in induced sputum: a feasibility study. *Am J Respir Crit Care Med* 1998;158:1178–1184.
 29. Palmenberg AC, Spiro D, Kuzmickas R, Wang S, Djikeng A, Rathe JA, Fraser-Liggett CM, Liggett SB. Sequencing and analyses of all known human rhinovirus genomes reveal structure and evolution. *Science* 2009;324:55–59.
 30. Greve JM, Davis G, Meyer AM, Forte CP, Yost SC, Marlor CW, Kamarck ME, McClelland A. The major human rhinovirus receptor is ICAM-1. *Cell* 1989;56:839–847.
 31. Staunton DE, Merluzzi VJ, Rothlein R, Barton R, Marlin SD, Springer TA. A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. *Cell* 1989;56:849–853.
 32. Tomassini JE, Graham D, DeWitt CM, Lineberger DW, Rodkey JA, Colonna RJ. cDNA cloning reveals that the major group rhinovirus receptor on HeLa cells is intercellular adhesion molecule 1. *Proc Natl Acad Sci USA* 1989;86:4907–4911.
 33. Hofer F, Gruenberger M, Kowalski H, Machat H, Huettinger M, Kuechler E, Blaas D. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. *Proc Natl Acad Sci USA* 1994;91:1839–1842.
 34. Lee WM, Kiesner C, Pappas T, Lee I, Grindle K, Jartti T, Jakiela B, Lemanske RF Jr, Shult PA, Gern JE. A diverse group of previously unrecognized human rhinoviruses are common causes of respiratory illnesses in infants. *PLoS ONE* 2007;2:e966.
 35. Khetsuriani N, Lu X, Teague WG, Kazerooni N, Anderson LJ, Erdman DD. Novel human rhinoviruses and exacerbation of asthma in children. *Emerg Infect Dis* 2008;14:1793–1796.
 36. Miller EK, Edwards KM, Weinberg GA, Iwane MK, Griffin MR, Hall CB, Zhu Y, Szilagyi PG, Morin LL, Heil LH, *et al.* A novel group of rhinoviruses is associated with asthma hospitalizations. *J Allergy Clin Immunol* 2009;123:98–104.
 37. Papadopoulos NG, Bates PJ, Bardin PG, Papi A, Leir SH, Fraenkel DJ, Meyer J, Lackie PM, Sanderson G, Holgate ST, *et al.* Rhinoviruses infect the lower airways. *J Infect Dis* 2000;181:1875–1884.
 38. Mosser AG, Brockman-Schneider R, Amineva S, Burchell L, Sedgwick JB, Busse WW, Gern JE. Similar frequency of rhinovirus-infectible cells in upper and lower airway epithelium. *J Infect Dis* 2002;185:734–743.
 39. Mosser AG, Vrtis R, Burchell L, Lee WM, Dick CR, Weisshaar E, Bock D, Swenson CA, Cornwell RD, Meyer KC, *et al.* Quantitative and qualitative analysis of rhinovirus infection in bronchial tissues. *Am J Respir Crit Care Med* 2005;171:645–651.
 40. Gerna G, Piralla A, Rovida F, Rognoni V, Marchi A, Locatelli F, Meloni F. Correlation of rhinovirus load in the respiratory tract and clinical symptoms in hospitalized immunocompetent and immunocompromised patients. *J Med Virol* 2009;81:1498–1507.
 41. Sorkness RL, Lee WM, Crisafi G, Vrtis R, Gern JE, Jarjour N. Rhinovirus-associated asthma exacerbations. *Am J Respir Crit Care Med* 2009;181:A5157.
 42. Denlinger LC, Kelly EA, Wolff M, Gaworski K, Liu LY, Guadarrama AG, Crisafi G, Lee WM, Vrtis R, Evans MD, *et al.* Nasal inflammatory response to viral infection in persons with and without asthma. *Am J Respir Crit Care Med* 2010;181:A5654.
 43. Jackson GG, Dowling HF, Spiesman IG, Boand AV. Transmission of the common cold to volunteers under controlled conditions. I. The common cold as a clinical entity. *AMA Arch Intern Med* 1958;101:267–278.
 44. Denlinger LC, Shi L, Guadarrama A, Schell K, Green D, Morrin A, Hogan K, Sorkness RL, Busse WW, Gern JE. Attenuated p2 × 7 pore function as a risk factor for virus-induced loss of asthma control. *Am J Respir Crit Care Med* 2009;179:265–270.
 45. Sorkness RL, Gonzalez-Fernandez G, Billmeyer EE, Evans MD, Gern JE, Jarjour NN. The asthma index: a continuous variable to characterize exacerbations of asthma. *J Allergy Clin Immunol* 2008;122:838–840.
 46. Lee WM, Grindle K, Pappas T, Marshall DJ, Moser MJ, Beaty EL, Shult PA, Prudent JR, Gern JE. High-throughput, sensitive, and accurate multiplex PCR-microsphere flow cytometry system for large-scale comprehensive detection of respiratory viruses. *J Clin Microbiol* 2007;45:2626–2634.
 47. Olenec JP, Kim WK, Lee WM, Vang F, Pappas TE, Salazar LE, Evans MD, Bork J, Roberg K, Lemanske RF Jr, *et al.* Weekly monitoring of children with asthma for infections and illness during common cold seasons. *J Allergy Clin Immunol* 2010;125:1001–1006.
 48. Bizzintino J, Lee WM, Laing IA, Vang F, Pappas T, Zhang G, Martin AC, Khoo SK, Cox DW, Geelhoed GC, *et al.* Association between human rhinovirus C and severity of acute asthma in children. *Eur Respir J* 2011;37:1037–1042.
 49. Bochkov YA, Palmenberg AC, Lee WM, Rathe JA, Amineva SP, Sun X, Pasic TR, Jarjour NN, Liggett SB, Gern JE. Molecular modeling, organ culture and reverse genetics for a newly identified human rhinovirus c. *Nat Med* 2011;17:627–632.
 50. Xiang Z, Gonzalez R, Wang Z, Xiao Y, Chen L, Li T, Vernet G, Paranhos-Baccala G, Jin Q, Wang J. Human rhinoviruses in Chinese adults with acute respiratory tract infection. *J Infect* 2010;61:289–298.
 51. Lau SK, Yip CC, Lin AW, Lee RA, So LY, Lau YL, Chan KH, Woo PC, Yuen KY. Clinical and molecular epidemiology of human rhinovirus C in children and adults in Hong Kong reveals a possible distinct human rhinovirus C subgroup. *J Infect Dis* 2009;200:1096–1103.
 52. Grunberg K, Sharon RF, Hiltermann TJ, Brahim JJ, Dick EC, Sterk PJ, Van Krieken JH. Experimental rhinovirus 16 infection increases intercellular adhesion molecule-1 expression in bronchial epithelium of asthmatics regardless of inhaled steroid treatment. *Clin Exp Allergy* 2000;30:1015–1023.
 53. Suzuki T, Yamaya M, Sekizawa K, Yamada N, Nakayama K, Ishizuka S, Kamanaka M, Morimoto T, Numazaki Y, Sasaki H. Effects of dexamethasone on rhinovirus infection in cultured human tracheal epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L560–L571.
 54. 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.
 55. Gustafson LM, Proud D, Hendley JO, Hayden FG, Gwaltney JM Jr. Oral prednisone therapy in experimental rhinovirus infections. *J Allergy Clin Immunol* 1996;97:1009–1014.
 56. Huynh ML, Malcolm KC, Kotaru C, Tilstra JA, Westcott JY, Fadok VA, Wenzel SE. Defective apoptotic cell phagocytosis attenuates prostaglandin E2 and 15-hydroxyeicosatetraenoic acid in severe

- asthma alveolar macrophages. *Am J Respir Crit Care Med* 2005;172:972-979.
57. Fitzpatrick AM, Holguin F, Teague WG, Brown LA. Alveolar macrophage phagocytosis is impaired in children with poorly controlled asthma. *J Allergy Clin Immunol* 2008;121:1372-1378.
58. Marshall DJ, Reisdorf E, Harms G, Beaty E, Moser MJ, Lee WM, Gern JE, Nolte FS, Shult P, Prudent JR. Evaluation of a multiplexed PCR assay for detection of respiratory viral pathogens in a public health laboratory setting. *J Clin Microbiol* 2007;45:3875-3882.
59. Covar RA, Szefer SJ, Zeiger RS, Sorkness CA, Moss M, Mauger DT, Boehmer SJ, Strunk RC, Martinez FD, Taussig LM. Factors associated with asthma exacerbations during a long-term clinical trial of controller medications in children. *J Allergy Clin Immunol* 2008;122:741-747.