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Similar Colds in Allergic Asthma Subjects and Non-Atopic Subjects After Inoculation with Rhinovirus-16

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Abstract

Background—Rhinovirus infections are frequent causes of asthma exacerbations.

Objective—This study was conducted to test whether subjects with and without allergic asthma have different responses to infection, and to identify baseline patient risk factors that predict cold outcomes.

Methods—Twenty mild persistent allergic asthma and 18 normal subjects were experimentally inoculated with type 16 rhinovirus. Subjects were evaluated at baseline, during the acute infection and during recovery for asthma and cold symptoms by validated questionnaire. Sputum and nasal lavage were evaluated for viral shedding, cytokines, and cellular inflammation.

Results—There were no group-specific significant differences in peak cold symptom score (10.0 ± 5.8 vs 11.1 ± 6.2 , asthma vs normal), peak nasal viral titers ($\log_{10} 4.3 \pm 0.8$ vs 3.7 ± 1.4 TCID₅₀/mL), or change in peak flow during the study ($10\% \pm 10\%$ vs $8\% \pm 6\%$). RV16 infection increased peak asthma index values in the asthma group (median 6→13, $p=0.003$), but only marginally in the normal group (median 4→7, $p=0.09$). More asthma subjects had detectable eosinophils in nasal lavage and sputum samples at baseline and during infection, but otherwise cellular and cytokine responses were similar. Baseline sputum eosinophilia and CXCL-8 (IL-8) levels were positively

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Clinical Implications:

Mild asthmatic and healthy control subjects had similar responses to rhinovirus after inoculation, but baseline measures such as sputum eosinophilia could predict individuals at risk for more severe infection.

Capsule Summary:

Subjects with mild asthma did not have more severe rhinovirus infections, nor shed greater amounts of virus. Mechanisms other than impaired antiviral responses may contribute to enhanced lower respiratory symptoms and exacerbations in asthma patients.

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associated with cold symptoms while CCL2 (MCP-1) levels were inversely associated with nasal viral shedding.

Conclusions—These findings suggest that mild allergic asthma and normal subjects have similar cold symptoms, inflammatory and antiviral responses. In addition, eosinophilia and other selective baseline measures of airway inflammation in subjects with or without asthma may predict respiratory outcomes with rhinovirus infection.

Keywords

Asthma; viral respiratory tract infections; virus-induced asthma; rhinovirus; eosinophil; CCL-2; CXCL-8; total serum IgE; allergy; common cold

INTRODUCTION

Viral respiratory infections, most commonly due to rhinoviruses (RV) are a principal cause of asthma exacerbations.^{1,2} In fact, asthma is an important risk factor for developing lower airway symptoms, including wheezing, from RV infection.³ Although the mechanisms for this are unclear, a leading hypothesis is that antiviral mechanisms are impaired in asthma. Increased RV replication and reduced interferon production were observed in primary bronchial epithelial cells from asthma compared to normal subjects.^{4,5} In contrast, studies utilizing experimental inoculation have demonstrated similar amounts of virus shed in nasal washes from subjects with and without asthma.^{6,7} These studies raise additional questions: is asthma associated with defective antiviral responses in epithelial cells, and if so, is this defect present in the upper airway, the lower airway, or both?

To test for asthma-related differences in antiviral responses, we experimentally inoculated both allergic asthma and non-atopic subjects to examine their responses to type 16 RV (RV16) infection. We hypothesized that abnormal host responses to RV16 in asthma subjects would lead to more severe infection and certain atopic and inflammatory predictors present at baseline increase the risk for more severe infection. We examined viral titers and RNA as well as inflammatory responses in nasal lavage, sputum and blood over the course of the infection. We also evaluated baseline blood cell responses and airway inflammatory indices to determine which host factors predict more severe viral infections.

METHODS

Subjects

Between August 2001 and November 2006, 445 subjects were recruited by advertisement and tested for RV16 serology, 151 had no detectable antibody to RV16, 60 were subsequently enrolled, and 38 (20 mild allergic asthma subjects and 18 normal controls) completed the study (Table I). Subjects did not complete the study for the following reasons: 12 withdrew consent, 7 lost to follow-up, and 3 withdrew for other reasons (too many medical problems, recurrent sinusitis, or recurrent colds that postponed the inoculation procedure).

The study included healthy adults between 18 and 45 years. The normal controls had negative skin prick testing to common aeroallergens, normal spirometry ($FEV_1 > 90\%$ predicted at baseline) with less than 5% improvement with beta agonist, and normal bronchial responsiveness to methacholine ($PC_{20} > 16$ mg/ml). Asthma subjects had a diagnosis of asthma for greater than six months, were allergic based on at least one positive skin prick to allergen testing (wheal size $>$ histamine control), were able to produce induced sputum, and had pre-bronchodilator $FEV_1 \geq 80\%$ predicted at baseline. Asthma subjects used only inhaled short-acting beta-agonist inhalers as needed and had bronchial hyperresponsiveness to methacholine

PC₂₀ ≤8 mg/ml and/or reversibility post-bronchodilator ≥12%. The methacholine challenge was performed by bronchial provocation techniques with diluent initially and then to increasing concentrations of methacholine (0.08, 0.16, 0.32, 0.63, 1.25, 2.5, 5, 10, and 25 mg/ml). From functional residual capacity to vital capacity with a five second breath hold, the subject inhaled 5 breaths of each concentration of methacholine administered by nebulization and spirometry was later performed.

At five minute intervals, dosing continued until at least a 20% decrease in FEV₁ was observed and then used to determine the PC₂₀ value by logarithmic interpolation. Subjects were administered a beta agonist if necessary. Exclusion criteria were the following: history of severe asthma exacerbations with respiratory infections, detectable serum RV-16 neutralizing antibody at screening, current smoker or more than 5 pack-year history, currently receiving immunotherapy, current use of nasal steroids, pregnant, breastfeeding, or currently participating in another clinical trial. All subjects provided informed consent, and the study was approved by University of Wisconsin Health Sciences Institutional Review Board.

Study design

At screening, subjects had a physical examination, allergy skin prick testing, blood draw for RV16-neutralizing antibody, urine pregnancy test, and pulmonary function tests before and after inhalation of albuterol. Specimens were collected at baseline, during the acute cold and upon recovery (Table I). Baseline assessments were performed 14 days prior and also 7 days prior to the first day of inoculation. These baseline measurements included sputum induction, nasal lavage, spirometry and blood draw for cytokines, PBMC proliferation, and WBC counts. When two samples were obtained for baseline measurements, the average value was utilized as the baseline value. Peak flow meters were issued at the beginning of the study and asthma and cold symptom diary cards were issued and data including peak flow measurements were collected at each visit. Nasal lavage was performed daily for the first 5 days starting at day 0 through day 4, then day 7 and 14. Sputum was induced on day 3, 7 and 14. Blood was drawn on day 0, 2, 7, 14 and 21. Subjects could use acetaminophen for discomfort, but were instructed not to use other cold medications that may mask their symptoms. Six asthma subjects and 2 normal subjects used acetaminophen during the acute cold ($p=0.25$, Fisher Exact Test). Data from 19 of these subjects related to detection of virus in the upper vs. lower airway have been previously published.⁸

Rhinovirus 16 Inoculation

None of the subjects had neutralizing antibody to RV16 at the time of screening, although nine subjects had low titers of RV16-neutralizing antibody detected in the serum obtained on the day of inoculation (mean 1.74, range 1–≥2.8). All baseline samples were tested for other respiratory viruses by multiplex PCR (Respiratory Viral Panel, EraGen BioSciences, Madison, WI).⁹ Molecular typing was then performed during the acute cold if baseline multiplex PCR was positive or if no RV16 was detected by tissue culture.¹⁰ RV16 inoculation was performed on two successive days with 1,000 50% tissue culture infective dose (TCID₅₀) units administered on each day by instilling 0.25 mL of RV16 suspension by pipette and by spraying 0.25 mL by atomizer into each nostril by previously described methods.¹¹

Nasal Lavage, Sputum Induction and Processing of Samples

Nasal lavage¹¹, sputum induction¹² and processing of samples¹² were performed as reported previously. Viral titers from nasal lavage were calculated after four tissue culture tubes containing MRC5 cells (human lung diploid cells, ViroMed, Minnetonka, MN) were inoculated for each serial 10-fold dilution of sample (10^0 – 10^{-7}) and incubated while rolling at 33°C for 10 days. Tubes were read at 24 hours and then every other day up to day 10.

TCID₅₀ was calculated as the concentration that was capable of infecting 50% of the tubes. Viral titers were expressed as TCID₅₀/mL.

Sputum induction and processing of samples were performed as reported earlier.¹² Sputum was induced after two puffs of albuterol and inhalation of 3% buffered saline solution mist for 20 minutes whereby subjects expectorated sputum as they felt they needed to and at serial time points of 10, 15 and 20 minutes. Cell counts and differentials were made from nasal lavage and sputum samples after treatment with 0.1% dithiothreitol. Supernatant fluids were stored at -80° C and later analyzed for cytokines.

Cytokine Analysis

Cytokines in nasal lavage samples were measured using Beadlyte Human Multi-Cytokine Flex Kits (Millipore, Temecula, CA). The sensitivities of the cytokine assays for IFN α 2, IFN γ , IL-6, CXCL8 (IL-8), IL-10, CCL2 (MCP-1), and CCL5 (RANTES) were 7 pg/mL, 2.3 pg/mL, 2.3 pg/mL, 2.3 pg/mL, 2.3 pg/mL, 6.9 pg/mL, and 4.1 pg/mL respectively. CXCL10 (IP-10) in serum was measured by ELISA (Upstate Biotechnology, Lake Placid, NY) with a lower limit of detection of 6.9 pg/mL.

PBMCs drawn at baseline visits were isolated from whole blood and incubated with RV16 to stimulate cytokine production. PBMCs (1 mL of 10⁶ cells/mL) in RPMI with 25% autologous plasma were incubated at 37°C and with 5% carbon dioxide for 6 days in polypropylene round-bottom 5-mL culture tubes with or without RV16 (1 mL samples, 10⁷ plaque-forming unit, PFU/mL). The cells were then pelleted and supernatants frozen (-80°C) for analysis.

Symptom Scores and Peak Asthma Index

Cold symptom assessments included cough, nasal discharge, sneezing, stuffy nose, sore throat, headache, malaise, chilliness, shaking chills, fever, laryngitis, aching joints or muscles, and watery or burning eyes and were measured daily on a scale (0=not present, 1=mild, 2=moderate, 3=severe) and recorded in diary form.¹³ The symptoms were scored four times daily, and the highest value for each symptom was used to calculate the total daily highest symptom score (TDHSS, maximum possible daily score=39).

The peak asthma index incorporated both subjective and objective measures and was calculated as previously described.¹⁴ Briefly, asthma symptom diaries were used to assess subjective symptoms such as chest tightness, wheeze, cough and shortness of breath, each rated on a scale of 0–3 and were recorded in the morning and evening for the first 21 days post-inoculation. These asthma symptoms along with nocturnal awakenings, number of puffs of albuterol and PEF were incorporated into the asthma index. The asthma index was computed as a moving average of 48 hours of data and the mean baseline values were subtracted. An asthma index of greater than 30 exceeds usual variability during stable periods, and is used as a criterion for loss of asthma control.

Viral RNA

Total RNA was extracted from samples (nasal lavage, sputum and sera) and reverse transcribed, and the quantity of RV RNA was measured using real-time PCR Applied Biosystems Prism 7000, (Foster City, CA) as reported previously.⁸ The standard curve was developed by extracting RV16 dilutions of known concentration ranging from 1 PFU to 1 × 10⁶ PFU; 1 PCR unit on the standard curve was defined as 1 PFU (~130 copies of RNA). Sera from 19 subjects were available and analyzed for viremia (viral RNA).

Total Serum IgE

Total IgE levels were measured on baseline serum samples by fluorenszyme immunoassay (FEIA) using an automated instrument (UniCAP 100; Phadia, Uppsala, Sweden) with a sensitivity of detection of 2 kU/L.

Statistical Analysis

Differences between allergic asthma and normal subjects were assessed using linear models, linear mixed-effects models, and logistic regression models. Relationships between peak cold severity measures (symptom scores and viral titers) and baseline predictors were examined using linear models that stratified by asthma diagnosis. Changes in cytokine levels from baseline to cold visits were assessed using linear mixed-effects models. Associations among sputum and nasal RV RNA and viral titers were summarized using partial correlation statistics which controlled for asthma diagnosis. Measurements that were below an assay's lower detection limit were addressed in one of two ways. When infrequent (<10% of observations), they were treated as a number below the limit but above zero. Otherwise the outcome measure was dichotomized as undetectable/detectable. Continuous outcome data were transformed for analysis, when needed, to obtain approximately normal distributions: viral titers and RV16 RNA were log-transformed, absolute cell counts and cytokine levels were either log-transformed or dichotomized where appropriate, and peak and mean TDHSS were square-root-transformed. Results were reported either as mean $\pm$ standard deviation, or for transformed data, median [25th percentile, 75th percentile]. A two-sided 5%-level test result was regarded as significant. An enrollment target of 20 allergic asthma and 20 normal subjects was chosen to provide 80% power to detect differences between the groups of the following estimated magnitudes: peak TDHSS, 5.6; day 3 TDHSS, 4.5; peak sputum RV16, 7-fold; peak nasal lavage RV16, 2.5-fold; peak nasal lavage viral titer, 6-fold; peak sputum neutrophils, 4-fold; and peak nasal lavage neutrophils, 2.5-fold.

RESULTS

Subject Characteristics

Twenty mild persistent allergic asthma subjects and 18 normal controls completed the study. There were no significant differences in ages or baseline FEV₁ of the two groups (Table II).

RV16 Infection—Thirty five of 38 subjects experienced an infection with RV16 as determined by viral shedding in nasal secretions. Three subjects from the normal group were not infected with RV16 (no detectable virus in nasal secretions). One of these subjects contracted a virus other than RV16 (RV88) after inoculation and was therefore excluded from the analysis. Unless otherwise stated, outcomes were analyzed on 37 (20 asthma and 17 normal) subjects.

Five asthma subjects had a virus detected in nasal lavage by RT-PCR at inoculation (1 RSV, 1 adenovirus, and 3 RV – 1 each with type W34, W21, and W9); these viruses were not detected by culture, and all subjects were asymptomatic. RSV and RV-W21 were detected also on day 1 and 2 while adenovirus remained detectable through day 14. The presence of virus at inoculation did not affect cold symptom scores or viral titers (data not shown).

Asthma Symptoms and Peak Flows—No one developed a severe exacerbation during this study that required oral corticosteroids, urgent or emergency room care, or hospitalization. There was a trend for higher peak asthma symptom scores in the asthma group (2.3 [1.2, 4.6] vs 0.5 [0, 2.8], $p=0.07$). The asthma index, which is a 48-hour rolling measure of subjective and objective data,¹¹ was evaluated for subjects with complete diary data (16 asthma and 14 normal subjects) during the first 21 days after inoculation. RV16 infection significantly

increased peak asthma index values in the asthma group (baseline median 6 [4.5, 10.8] with increase to 13 [8.3, 21.8], $p=0.003$), but only marginally in the normal group (baseline median 4 [2.5, 6] with increase to 7 [2, 8], $p=0.09$, Figure 1). Three subjects in the asthma group experienced an asthma index greater than 30 which was defined by Sorkness et al as exceeding usual variability and the threshold for defining an asthma exacerbation.¹⁴

Complete baseline PEF were obtained from 18 asthma and 15 normal subjects. Peak expiratory flow rates (PEF) during the acute cold were similar in both groups and also within groups over the course of the infection. On day 2 after inoculation, the morning peak flows of asthma subjects were $95\% \pm 10\%$ of baseline compared with normal subjects $99\% \pm 5\%$ ($p=0.30$). The minimum post-inoculation PEF were also similar ($90\% \pm 10\%$ vs $92\% \pm 6\%$) as was the maximum change in peak flow from baseline ($10\% \pm 10\%$ vs $8\% \pm 6\%$).

Cold Symptoms—Subjects in both groups developed similar cold symptoms over the first 7 days (means $\pm$ SD: asthma 4.8 ± 3.4 vs normal 6.0 ± 3.7), and symptom scores were greatest on the second day after inoculation (7.7 ± 5.6 vs 7.8 ± 6.3 , Figure 2A). There were no significant differences between asthma and normal subjects in peak cold symptom scores (10.0 ± 5.8 vs 11.1 ± 6.2 , $p=0.29$).

We classified subjects based on their peak cold symptom score and defined them as having a cold if their peak cold symptom score was equal or greater than 4. Based on this criteria, 17 of 20 asthma and 16 of 17 normal subjects experienced a clinically significant cold. We further categorized subjects based on the severity of their cold (4–6 mild, 7–10 moderate, ≥ 11 severe) and found similar frequencies between the groups [(mild: 5 asthma vs 3 normal subjects), (moderate: 5 vs 5), and (severe 7 vs 8)]. There was no significant relationship between peak cold symptoms and the peak asthma index in subjects with asthma.

Viral shedding

Infectious virus: Subjects in both the asthma and normal groups had similar viral shedding in nasal secretions during the RV infection. Both the asthma and normal groups had highest viral shedding on day 3 (Figure 2B). There were no significant differences in peak viral titers ($\log_{10}$ 4.3 ± 0.8 vs 3.7 ± 1.4 TCID₅₀/mL). Upon resolution of the acute infection (2 weeks post-inoculation), there was a trend for more asthma subjects to have persistently detectable virus (60% vs 31%, $p=0.06$). Peak viral titers were associated with peak symptom scores ($r=0.45$, $p=0.005$) and peak nasal neutrophils ($r=0.61$, $p<0.0001$), but not peak sputum neutrophils ($r=0.13$, $p=0.46$).

RV16 RNA: Both asthma and normal subjects had similar RV RNA levels in nasal lavage and sputum throughout the duration of the infection (Figure 2C and 2D). RV16 RNA in nasal lavage had the highest value on the third day post-inoculation (asthma $n=17$ vs. normal $n=13$: $\log_{10}$ 4.7 ± 0.7 vs 4.4 ± 1.1 PCR units) with decreasing amounts at 1 week (asthma $n=17$ vs. normal $n=11$: $\log_{10}$ 3.3 ± 0.8 vs 3.8 ± 0.9 PCR units) and at 2 weeks (asthma $n=16$ vs. normal $n=10$: $\log_{10}$ 1.8 ± 1.5 vs 1.4 ± 1.9 PCR units) post-inoculation. The highest RV16 RNA in sputum occurred on the third day post-inoculation (asthma $n=18$ vs. normal $n=11$: $\log_{10}$ 3.5 ± 1.0 vs 3.6 ± 1.7 PCR units). There were no significant differences noted between the groups.

During the acute cold (day 3), RV RNA levels in nasal lavage were positively associated with RV RNA levels in sputum ($n=25$, $r=0.59$, $p=0.003$). RV RNA levels from nasal lavage and sputum were positively associated with nasal lavage quantitative viral cultures ($n=30$, $r=0.79$, $p<0.0001$ and $n=29$, $r=0.55$, $p=0.003$, respectively).

Cellularity—In sputum, subjects with asthma ($n=19$, normal $n=15$) were more likely to have detectable eosinophils at baseline (detectable: 56% vs 8%, $p=0.008$; 1.2 [1.0, 6.0] vs 0.0 [0.0,

$0.9] \times 10^3$ cells/g), acute infection (82% vs 33%, $p=0.04$; $3.0 [1.3, 8.0]$ vs $0.4 [0.0, 1.3]$), and recovery (72% vs 33%, $p=0.14$; $3.2 [0.9, 7.8]$ vs $0.4 [0.0, 1.0]$). In nasal lavage, more subjects with asthma had detectable eosinophils at baseline (63% vs 31%, $p=0.10$; $0.1 [0.0, 0.3]$ vs $0.0 [0.0, 0.0] \times 10^3$ cells/g), acute infection (80% vs 27%, $p=0.004$; $0.9 [0.2, 9.2]$ vs $0.0 [0.0, 0.1]$), and recovery (74% vs 27%, $p=0.04$; $0.7 [0.0, 5.7]$ vs $0.0 [0.0, 0.0]$).

There were minimal group-specific differences in other types of cells in sputum and nasal lavage. In sputum, the numbers of neutrophils were similar between groups at baseline (asthma $282 [196, 380]$ vs normal $166 [116, 237] \times 10^3$ cells/g), acute infection ($384 [144, 566]$ vs $169 [134, 501]$), and recovery ($202 [177, 639]$ vs $128 [68, 214]$) (asthma $n=19$, normal $n=15$, supplement Figure 1A). Sputum neutrophils increased during the acute cold. No significant changes in either sputum monocytes or lymphocytes were noted over the course of the infection (supplement Figure 1B and 1C). In nasal lavage samples, neutrophils, monocytes, and lymphocytes increased during the acute cold, but there were no differences between the two groups (Supplement Figure 2A, 2B and 2C).

Cytokines in Nasal Lavage—In nasal lavage samples, the levels of several cytokines (IL-6, IL-10, CXCL8, CCL2, and CCL5) increased significantly during the acute infection compared with baseline levels ($p<0.001$, Supplement Figure 3). Peak CXCL8 levels were associated with peak nasal neutrophils ($r=0.67$, $p<0.0001$). IFN $\alpha 2$ and IFN γ responses were detectable in only about 1/3 of subjects during the acute cold and levels significantly increased during acute infection compared to baseline ($p<0.001$). Throughout the study, there was no consistent difference between asthma and normal subjects with detectable IFN $\alpha 2$ or IFN γ . At baseline, more asthma than control subjects had detectable CCL2 in nasal lavage samples (47% vs 12% $p=0.04$). There were no other group-specific differences in nasal lavage fluid cytokines during the course of infection.

Serum CXCL10 and RV RNA—Serum levels of CXCL10 were assessed during the course of the acute infection. Subjects had similar CXCL10 levels at baseline (asthma 210 ± 156 pg/mL vs. normal 152 ± 108 pg/mL). After inoculation, all subjects had an approximately 3-fold increase in serum CXCL10 ($p<0.0001$); both groups had a wide range of responses with no group-specific differences ($p=0.55$). CXCL10 levels returned to baseline levels upon resolution of the cold (14 days post-inoculation). Additional sera from 19 subjects were available and analyzed for viremia (RV16 RNA), however, none was detected.

RV16-induced cytokine production in PBMCs—PBMCs were obtained from asthma and control subjects at baseline and incubated with RV16 (1×10^7 PFU/mL) to evaluate ex vivo cytokine response. Asthma ($n=16$) and control ($n=16$) subjects had similar cytokine responses: IFN $\alpha 2$ (asthma $7.0 [2.6, 13.9]$ vs normal $7.6 [2.6, 12.2] \times 10^2$ pg/mL), IFN γ ($6.6 [3.9, 12.1]$ vs $9.6 [5.5, 16.3]$), IL-6 ($5.6 [3.4, 17.7]$ vs $18.3 [7.1, 36.0]$) and IL-10 ($1.1 [0.6, 1.6]$ vs $1.1 [0.5, 2.1]$). There were no group-specific differences in RV16-induced cytokine responses.

Predictors of Cold Severity—Baseline data were evaluated as predictors of cold severity. Subjects, with or without asthma, with detectable sputum eosinophils (≥ 1 eosinophil in 300 WBC) before inoculation reported more severe cold symptoms ($p=0.05$, Figure 3A). Subgroup analysis indicated that this was true both within the asthma and control groups (Figure 3B). Baseline eosinophilia was not related to viral titers or RNA in airway secretions during the cold.

In nasal lavage fluid obtained at baseline ($n=36$), CCL2 levels were inversely related to peak viral titer ($\log_{10}$ TCID $_{50}$ /mL: undetectable 4.2 ± 1.1 vs detectable 3.7 ± 1.4 , $p=0.03$), and there was a trend for an association between baseline CXCL8 levels and peak cold symptom scores

(TDHSS: undetectable 9.5 ± 5.5 vs detectable 11.9 ± 6.5 , $p=0.08$). Baseline levels of other nasal lavage cytokines had no relationship with either peak cold symptom score or peak viral titer.

In baseline PBMC responses ($n=32$), there was a trend for an inverse relationship between RV16-induced IFN α 2 secretion and peak cold symptoms scores ($r=-0.35$, $p=0.07$). Other PBMC cytokine responses (IFN γ , IL-6 and IL-10) were not related to peak cold symptom score or peak viral titer.

Total IgE levels were greater in the asthma group, but did not predict increased cold symptoms or higher peak viral titers during the experimental cold. Although none of the subjects at screening had neutralizing antibody to RV16, nine subjects on the day of inoculation had low titers of RV16-neutralizing antibody in serum. Low level antibody present at inoculation was associated with lower peak viral titer (detectable 3.1 ± 1.6 vs. undetectable $4.3 \pm 0.8 \log_{10}$ TCID $_{50}$ /mL, $p=0.008$), but was not related to peak symptom score (detectable 10.9 ± 6.0 vs undetectable 9.2 ± 5.9 , $p=0.56$).

DISCUSSION

Rhinovirus infections cause greater morbidity in individuals with asthma.^{15,16} A proposed mechanism for this difference is that reduced interferon responses in epithelial cells lead to enhanced viral replication, and ultimately increased respiratory symptoms.^{4,5} In this experimental inoculation study, we found that subjects with and without mild allergic asthma had similar cold symptoms and viral shedding in nasal lavage and sputum. RV16 infection caused a modest increase in the peak asthma index only in asthma subjects, and none of the subjects with asthma experienced a severe exacerbation. With the exception of increased eosinophils in asthma, the cytokine and cellular composition of the nasal lavage and sputum samples were similar between the asthma and normal groups. Certain baseline characteristics, such as sputum eosinophils and CXCL8 in nasal lavage, were positively associated with symptoms, but these relationships were not specific to asthma. Together, these findings do not support the hypothesis that viral replication is enhanced in subjects with mild allergic asthma, and instead provide evidence that individual variations in airway inflammatory characteristics could influence cold severity.

Our results are similar to and extend those of previous experimental inoculation studies that found no differences between normal subjects and those with asthma in symptoms¹⁷ or viral shedding in the upper airway,⁶ as determined by viral culture scores. A more recent experimental inoculation study found asthma subjects had greater lower respiratory symptoms and reduced lung function, but no significant increase in viral shedding in the nasal secretions or sputum.⁷ The finding of similar viral shedding seems contrary, however, to two previous studies conducted *in vitro* with isolated epithelial cells.^{4,5} Although the reason for the discordant findings is unknown, it is possible that reduced epithelial IFN responses are limited to more severe asthma subjects than were included in our study. Additional studies across the spectrum of asthma severity are required to address this possibility. Another potential explanation for this discrepancy is that cells *in vitro* may behave differently than cells *in vivo*, perhaps due to multicellular interactions that are necessary for peak antiviral responses.¹⁸

Group-specific differences in the immune response to rhinovirus infection were minor. One exception is that eosinophils were increased in the sputum and nasal secretions of subjects with asthma at baseline. In nasal lavage samples, eosinophils remained increased 2 weeks post inoculation, and this is similar to the prolonged nasal eosinophilia observed in allergic patients after natural colds.¹⁹ We did observe increases in neutrophils, CXCL8, and other cytokines

during the acute cold compared to baseline, as reported in previous studies,^{7,11} but no group specific differences.

A secondary objective of this study was to identify baseline variables that predict respiratory outcome in viral illnesses. We found that subjects, either asthma or control, with detectable sputum eosinophils reported more severe symptoms, and this relationship was independent of effects on viral replication. These findings are intriguing as Rakes et al found that RV infection and airway eosinophilia synergistically increased the risk for wheezing in children.²⁰ Collectively, these results suggest that eosinophils either contribute to airway inflammation or symptoms in the context of RV infection. In accordance with this hypothesis, Davoine *et al* recently reported that RV-induced activation of combined antigen presenting cells and CD4+ T cells could in turn cause eosinophil degranulation.²¹ In previous experimental inoculation studies, RV infections were found to enhance allergen-induced eosinophil recruitment,²² however, it is puzzling that induction of eosinophils by allergen challenge before viral inoculation did not increase the severity of illness.²³ A more recent experimental inoculation study found that greater numbers of eosinophils in BAL from asthma subjects were significantly related to a larger maximal fall in PEF.⁷ In that same study, the greater number of sputum eosinophils correlated also with greater total lower respiratory symptoms as well as a decrease in PC₁₀ histamine, reflecting greater bronchial responsiveness.⁷ Furthermore, eosinophils may have some antiviral activity.²⁴ Additional studies are needed to clarify relationships, which appear to be complex, between airway eosinophils and RV illnesses.

Other predictors of respiratory outcomes included baseline levels of airway cytokines and cytokine responses of PBMC. Baseline CCL2 levels from nasal samples were inversely related to peak viral titer, but not symptom score. CCL2 is secreted by RV-stimulated alveolar macrophages or blood monocytes in vitro,²⁵ and higher CCL2 responses could recruit more monocytes to the airway to bolster antiviral immune responses. In addition, there was a trend for a positive association between baseline CXCL8 levels in nasal secretions and higher peak symptom scores. CXCL8 is secreted in response to a number of insults, including pollutants, allergens, and oxidative stress. Therefore, it is possible that baseline levels of CXCL8 are indicative of exposure to other factors that damage the epithelium, and thereby promote more severe illnesses.²⁶

There was a trend for an inverse relationship between IFN α 2 responses in PBMC and peak cold symptom scores ($r=-0.35$, $p=0.07$). Unlike previous studies conducted in allergic and normal subjects without asthma,²⁷ and in asthma and normal volunteers,⁷ we found no association between IFN- γ responses and viral outcomes. We also found no association with IL-10 as was recently reported, albeit with caution due to small sample size.⁷

RV16-specific antibody titers were assessed twice: once at screening and again on the day of inoculation. Subjects with low titers of RV16-neutralizing antibody at inoculation had lower peak viral titers compared with those with absent titers. The change in titer during a relatively short period of time may represent variability of the assay, or else interim exposure to RV16 or an antigenically related virus. Notably, the main results of the study were not altered after controlling for effects of the neutralizing antibody. In addition, viral detection at the time of inoculation among asymptomatic subjects had no appreciable effects on cold symptoms or RV16 shedding in our study.

Our study, which utilized experimental inoculation, has a number of strengths and some limitations. Standardized inoculation procedures limit the variability related to the infection, and thus allow detailed examination of host-specific factors that influence viral shedding and symptom severity. One limitation of the study is that we recruited subjects who had mild asthma that was relatively stable. Responses to infections are quite likely to be different in patients

with poorly controlled asthma, and therefore the ability to generalize our findings to all patients is limited. Additionally, asthma is a diverse disease, and it is possible that there are subgroups of mild asthma associated with impaired antiviral responses. Furthermore, our study design allowed for observations related to cold severity and associated inflammation, but not exacerbations, which are unusual after experimental inoculation. In this study, three subjects in the asthma group experienced an asthma index greater than 30 which was defined by Sorkness et al¹⁴ as exceeding usual variability and the threshold for defining an asthma exacerbation; however, none of them required oral corticosteroids, urgent or emergency room care, or hospitalization indicative of a severe exacerbation. The sample size of this study was sufficient to have 80% power to detect a one log difference in viral shedding, and larger studies would be needed to detect smaller differences. Finally, it is possible that there are some strains of RV, other than RV16, that could have greater differential effects on asthma.

Overall, we found that cold severity, inflammation, and viral shedding were similar between mild allergic asthma subjects and non-atopic control subjects. Since even mild asthma is associated with an increased risk of virus-induced wheezing, these findings suggest that there may be other mechanisms, independent of viral replication, that contribute to enhanced lower respiratory symptoms. Furthermore, our findings together with clinical observations suggest the possibility that baseline sputum eosinophilia promotes more severe infections. Identifying the precise mechanisms for RV infections to cause more severe lower airway manifestations in asthma is a necessary step towards the development of new strategies for reducing virus-induced asthma morbidity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

FEV₁	Forced expiratory volume in the first second
IFN	Interferon
IgE	Immunoglobulin E
IL-8	Interleukin 8, CXCL8
IP-10	IFN γ -inducible protein 10, CXCL10
MCP-1	Monocyte chemotactic protein-1, CCL2
PBMC	Peripheral blood mononuclear cell
PC₂₀	

	Provocation concentration of inhaled methacholine required to reduce FEV1 by 20%
PCR	Polymerase chain reaction
PEF	Peak expiratory flow
PFU	Plaque-forming unit
RANTES	Regulated on activation, normal T-cell expressed and secreted, CCL5
RSV	respiratory syncytial virus
RV	Rhinovirus
RV16	Rhinovirus strain 16
TCID₅₀	Tissue culture infective dose 50%
TDHSS	Total daily highest symptom score

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Figure 1.

Peak asthma index of subjects during baseline (BSLN) and cold (COLD) periods in subjects with allergic asthma (●, filled circles) and those without asthma (○, open circles). The dotted line differentiates subjects with and without exacerbations, and the bars indicate medians for each group. There was a significant increase in median values from baseline to cold in the asthma group (asterisk *, $p=0.003$), but not in the control group ($p=0.09$). (Figure is adapted from Fig. 2, reference # ¹⁴.)

Figure 2.

Group specific outcomes of experimental infection. During the RV16 infection, (A) cold symptom scores, (B) nasal lavage viral titers, (C) sputum RV RNA, and (D) nasal RV RNA in normal, (○, open circles n=17) and allergic asthma (●, filled circles n=20) subjects. Subjects were inoculated on days 0 and 1. All data are expressed as mean ± sd. The day 0 sputum values were obtained from samples obtained at the baseline visits.

Figure 3.

Relationship of sputum eosinophils to cold symptoms. (A) At baseline, subjects were determined to have detectable (≥ 1 eosinophil per 300 WBC in sputum) or undetectable sputum eosinophils. Subjects with detectable sputum eosinophils at baseline had higher peak cold symptom score with RV16 infection ($p=0.05$). The box represents the 25th percentile, median, and 75th percentile; and the whiskers illustrate the 5th and 95th percentiles. (B) Subgroup analysis revealed that subjects with sputum eosinophils even without asthma tended to have worse cold symptoms, but this was not statistically significant.

Table 1

Study Design

	Baseline		Acute Cold							Convalescence			
Visit	1	2	3	4	5	6	7	8	9	10			
Day	-14	-7	0	1	2	3	4	7	14	21			
RV16 Inoculation			X	X									
Nasal Lavage		X	X	X	X	X	X	X	X				
Sputum Induction	X	X				X		X	X				
Blood Draw	X	X	X		X			X	X	X			
Spirometry		X	X	X	X	X	X	X	X				
Methacholine	X												

Table II

Baseline Characteristics

Characteristics	Allergic Asthma (n=20)	Normal (n=17)	p-value
Gender (F:M)	13:7	12:5	0.99
Age (y)	22.3 ± 4.1	24.2 ± 7.3	0.33
FEV1 (L)	4.02 ± 0.75	3.97 ± 0.66	0.82
FEV1 (% predicted)	100 ± 9	102 ± 13	0.57
Methacholine PC ₂₀ Median (range)	1.415 (0.13-7)	>16	-----
Positive Skin Tests ([#])	5.1 ± 2.0	0 ± 0	-----
Total IgE (kU/L)	258 ± 244	45 ± 108	<0.0001

Mean ± standard deviation

F, female. M, male. y, years. L, liter. PC₂₀ provocative concentration of methacholine causing a 20% fall in FEV1.

[#], number.