

Similar colds in subjects with allergic asthma and nonatopic subjects after inoculation with rhinovirus-16

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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 subjects with mild persistent allergic asthma and 18 healthy subjects were experimentally inoculated with rhinovirus-16. Subjects were evaluated at baseline, during the acute infection, and during recovery for asthma and cold symptoms by using a validated questionnaire. Sputum and nasal lavage fluid were evaluated for viral shedding, cytokines, and cellular inflammation.

Results: There were no group-specific significant differences in peak cold symptom scores (10.0 ± 5.8 vs 11.1 ± 6.2 , asthmatic vs healthy subjects), peak nasal viral titers ($\log_{10} 4.3 \pm 0.8$ vs 3.7 ± 1.4 50% tissue culture infective dose/mL, respectively), or changes in peak flow during the study ($10\% \pm 10\%$ vs $8\% \pm 6\%$, respectively). Rhinovirus-16 infection increased peak asthma index values in the asthmatic group (median, 6 → 13; $P = .003$) but only marginally in the healthy group (median, 4 → 7; $P = .09$). More asthmatic 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 CXCL8 (IL-8) levels were positively associated with cold symptoms, whereas CCL2 (monocyte chemoattractant protein 1) levels were inversely associated with nasal viral shedding.

Conclusions: These findings suggest that subjects with mild allergic asthma and healthy subjects have similar cold symptoms and inflammatory and antiviral responses. In

addition, eosinophilia and other selective baseline measures of airway inflammation in subjects with or without asthma might predict respiratory outcomes with rhinovirus infection. (J Allergy Clin Immunol 2009;123:1000-1008.)

Key words: Asthma, viral respiratory tract infections, virus-induced asthma, rhinovirus, eosinophil, CCL2, CXCL8, total serum IgE, allergy, common cold

Viral respiratory tract infections, most commonly caused by rhinoviruses, are a principal cause of asthma exacerbations.^{1,2} In fact, asthma is an important risk factor for the development of lower airway symptoms, including wheezing, from rhinovirus infection.³ Although the mechanisms for this are unclear, a leading hypothesis is that antiviral mechanisms are impaired in asthma. Increased rhinovirus replication and reduced interferon production were observed in primary bronchial epithelial cells from asthmatic compared with healthy subjects.^{4,5} In contrast, studies using 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 subjects with allergic asthma and nonatopic subjects to examine their responses to rhinovirus-16 (RV16) infection. We hypothesized that abnormal host responses to RV16 in asthmatic subjects would lead to more severe infection and that 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 fluid, 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 subjects with mild allergic asthma and 18 healthy control subjects) completed the study. Subjects did not complete the study for the following reasons: 12 withdrew consent, 7 were 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 of age. The healthy control subjects had negative skin prick test responses to common aeroallergens,

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Abbreviations used

PEF: Peak expiratory flow
 PFU: Plaque-forming units
 RV16: Rhinovirus-16
 TCID₅₀: 50% Tissue culture infective dose
 TDHSS: Total daily highest symptom score

normal spirometry (FEV₁ > 90% of predicted value at baseline) with less than 5% improvement with β -agonists, and normal bronchial responsiveness to methacholine (PC₂₀ > 16 mg/mL). Asthmatic subjects had a diagnosis of asthma for greater than 6 months, were allergic based on at least 1 positive skin prick test response to allergen (wheal size greater than that elicited by the histamine control), were able to produce induced sputum, and had prebronchodilator FEV₁ values of 80% or more of the predicted value at baseline. Asthmatic subjects used only inhaled short-acting β -agonist inhalers as needed and had bronchial hyperresponsiveness to methacholine PC₂₀ of 8 mg/mL or less, reversibility after bronchodilator of 12% or greater, or both. The methacholine challenge was performed by using bronchial provocation techniques with diluent initially and then 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 5-second breath hold, the subject inhaled 5 breaths of each concentration of methacholine administered by means of a nebulizer, and spirometry was later performed. At 5-minute intervals, dosing continued until at least a 20% decrease in FEV₁ was observed and then used to determine the PC₂₀ value by means of logarithmic interpolation. Subjects were administered a β -agonist if necessary. Exclusion criteria were the following: a history of severe asthma exacerbations with respiratory infections, detectable serum RV16 neutralizing antibody at screening, current smoking or a more than 5 pack-year history, current use of immunotherapy, current use of nasal steroids, pregnancy, breast-feeding, or current participation in another clinical trial. All subjects provided informed consent, and the study was approved by the University of Wisconsin Health Sciences Institutional Review Board.

Study design

At screening, subjects underwent a physical examination, allergy skin prick testing, blood draw for RV16-neutralizing antibody, a urine pregnancy test, and pulmonary function tests before and after inhalation of albuterol. Specimens were collected at baseline, during the acute cold, and on recovery (Table I). Baseline assessments were performed 14 days before and also 7 days before the first day of inoculation. These baseline measurements included sputum induction, nasal lavage, spirometry, and blood draw for cytokines, PBMC proliferation, and white blood cell counts. When 2 samples were obtained for baseline measurements, the average value was used as the baseline value. Peak flow meters were issued at the beginning of the study. 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 and then day 7 and 14. Sputum was induced on days 3, 7, and 14. Blood was drawn on days 0, 2, 7, 14, and 21. Subjects could use acetaminophen for discomfort but were instructed not to use other cold medications that might mask their symptoms. Six asthmatic subjects and 2 healthy subjects used acetaminophen during the acute cold ($P = .25$, Fisher exact test). Data from 19 of these subjects related to detection of virus in the upper versus lower airways have been previously published.⁸

RV16 inoculation

None of the subjects had neutralizing antibody to RV16 at the time of screening, although 9 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 using multiplex PCR (Respiratory Viral Panel; EraGen BioSciences, Madison, Wis).⁹ Molecular typing was then performed during the acute cold if baseline multiplex PCR results were positive or if no RV16 was detected by means of tissue culture.¹⁰ RV16 inoculation

was performed on 2 successive days with 1000 50% tissue culture infective dose (TCID₅₀) units administered on each day by instilling 0.25 mL of RV16 suspension with a pipette and by spraying 0.25 mL through an atomizer into each nostril according to previously described methods.¹¹

Nasal lavage, sputum induction, and processing of samples

Nasal lavage,¹¹ sputum induction,¹² and processing of samples¹² were performed as reported previously. Viral titers from nasal lavage fluid were calculated after 4 tissue-culture tubes containing MRC5 cells (human lung diploid cells; ViroMed, Minnetonka, Minn) were inoculated for each serial 10-fold dilution of sample (10^0 to 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 per milliliter.

Sputum induction and processing of samples were performed as reported earlier.¹² Sputum was induced after 2 puffs of albuterol and inhalation of 3% buffered saline solution mist for 20 minutes, whereby subjects expectorated sputum as they believed they needed 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 with Beadlyte Human Multi-Cytokine Flex Kits (Millipore, Temecula, Calif). The sensitivities of the cytokine assays for IFN- α 2, IFN- γ , IL-6, CXCL8 (IL-8), IL-10, CCL2 (monocyte chemoattractant protein 1), and CCL5 (RANTES) were 7, 2.3, 2.3, 2.3, 2.3, 6.9, and 4.1 pg/mL, respectively. CXCL10 (interferon-inducible protein 10) in serum was measured by means of 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 10^6 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, 10^7 plaque-forming units [PFU]/mL). The cells were then placed in pellets, and supernatants were 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 from 0 to 3 (0, not present; 1, mild; 2, moderate; and 3, severe) and recorded in diary form.¹³ The symptoms were scored 4 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 to 3, and were recorded in the morning and evening for the first 21 days after inoculation. These asthma symptoms, along with nocturnal awakenings, number of puffs of albuterol, and peak expiratory flow (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 the 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 fluid, sputum, and sera) and reverse transcribed, and the quantity of rhinovirus RNA was measured by using real-time PCR (Prism 7000; Applied Biosystems, Foster City, Calif), as reported previously.⁸ The standard curve was developed by extracting RV16 dilutions of known concentrations ranging from 1 to 1×10^6 PFU; 1 PCR unit on

TABLE I. 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	Subjects with allergic asthma (n = 20)	Healthy subjects (n = 17)	P value
Sex (F/M)	13/7	12/5	.99
Age (y)	22.3 ± 4.1	24.2 ± 7.3	.33
FEV ₁ (L)	4.02 ± 0.75	3.97 ± 0.66	.82
FEV ₁ (% predicted)	100 ± 9	102 ± 13	.57
Methacholine PC ₂₀ , median (range)	1.415 (0.13-7)	>16	—
Positive skin test results (no.)	5.1 ± 2.0	0 ± 0	—
Total IgE (kU/L)	258 ± 244	45 ± 108	<.0001

Values are presented as means ± SDs.

F, Female; M, male.

the standard curve was defined as 1 PFU (approximately 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 using the fluorezyme immunoassay with an automated instrument (UniCAP 100; Phadia, Uppsala, Sweden) with a sensitivity of detection of 2 kU/L.

Statistical analysis

Differences between subjects with allergic asthma and healthy subjects were assessed by 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 by using linear models that were stratified by asthma diagnosis. Changes in cytokine levels from baseline to cold visits were assessed by using linear mixed-effects models. Associations among sputum and nasal rhinovirus RNA and viral titers were summarized by using partial correlation statistics, which controlled for asthma diagnosis. Measurements that were less than an assay's lower detection limit were addressed in one of 2 ways. When infrequent (<10% of observations), they were treated as a number less than the limit but greater than 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 values were square root transformed. Results were reported either as means ± SDs or, for transformed data, medians [25th-75th percentiles]. A 2-sided 5% level test result was regarded as significant. An enrollment target of 20 subjects with allergic asthma and 20 healthy 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

Subjects' characteristics

Twenty subjects with mild persistent allergic asthma and 18 healthy control subjects completed the study. There were no significant differences in ages or baseline FEV₁ values of the 2 groups (Table II).

RV16 infection

Thirty-five of 38 subjects experienced an infection with RV16, as determined by means of viral shedding in nasal secretions. Three subjects from the healthy 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 for 37 (20 asthmatic and 17 healthy) subjects.

Five asthmatic subjects had a virus detected in nasal lavage fluid by means of RT-PCR at inoculation (1 had respiratory syncytial virus, 1 had adenovirus, and 3 had rhinoviruses, 1 each of type W34, W21, and W9); these viruses were not detected by means of culture, and all subjects were asymptomatic. Respiratory syncytial virus and rhinovirus W21 were detected also on days 1 and 2, whereas adenovirus remained detectable through day 14. The presence of a virus at inoculation did not affect cold symptom scores or viral titers (data not shown).

Asthma symptoms and peak flows

No one had a severe exacerbation during this study that required oral corticosteroids, urgent or emergency department care, or hospitalization. There was a trend for higher peak asthma symptom scores in the asthmatic group (2.3 [25th-75th percentile, 1.2-4.6] vs 0.5 [25th-75th percentile, 0-2.8], $P = .07$). The asthma index, which is a 48-hour rolling measure of subjective and objective data,¹⁴ was evaluated for subjects with complete diary data (16 asthmatic and 14 healthy subjects) during the first 21 days after inoculation. RV16 infection significantly increased peak asthma index values in the asthma group (baseline median of 6 [25th-75th percentile, 4.5-10.8] with an increase to 13 [25th-75th percentile, 8.3-21.8], $P = .003$) but only marginally in the healthy group (baseline median of 4 [25th-75th percentile, 2.5-6] with an increase to 7 [25th-75th percentile, 2-8], $P = .09$, Fig 1). Three subjects in the asthmatic group experienced an asthma index of greater than 30, which was defined by Sorkness

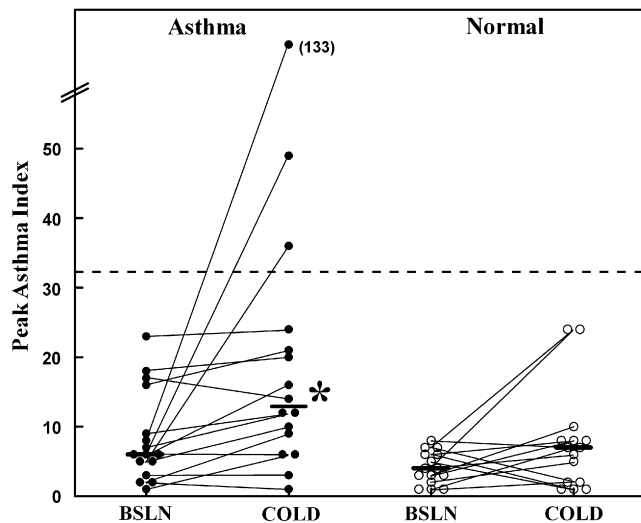


FIG 1. Peak asthma index of subjects during baseline (BSLN) and cold (COLD) periods in subjects with allergic asthma (solid circles) and those without asthma (open circles). The dotted line differentiates subjects with and without exacerbations, and the bars indicate medians for each group. There was a significant increase in median values from baseline to cold periods in the asthma group (* $P = .003$) but not in the control group ($P = .09$). Adapted from Fig 2 from Sorkness et al.¹⁴

et al¹⁴ as exceeding the usual variability and the threshold for defining an asthma exacerbation.

Complete baseline PEF rates were obtained from 18 asthmatic and 15 healthy subjects. PEF rates 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 flow rates of asthmatic subjects were $95\% \pm 10\%$ of baseline values compared with values seen in healthy subjects ($99\% \pm 5\%$, $P = .30$). The minimum postinoculation PEF rates 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 had similar cold symptoms over the first 7 days (mean $\pm$ SD: 4.8 ± 3.4 for asthmatic subjects vs 6.0 ± 3.7 for healthy subjects), and symptom scores were greatest on the second day after inoculation (7.7 ± 5.6 vs 7.8 ± 6.3 , respectively; Fig 2, A). There were no significant differences between asthmatic and healthy subjects in peak cold symptom scores (10.0 ± 5.8 vs 11.1 ± 6.2 , respectively; $P = .29$).

We classified subjects based on their peak cold symptom scores and defined them as having a cold if their peak cold symptom score was equal to or greater than 4. Based on these criteria, 17 of 20 asthmatic and 16 of 17 healthy subjects experienced a clinically significant cold. We further categorized subjects based on the severity of their cold (4-6 for mild, 7-10 for moderate, and ≥ 11 for severe symptoms) and found similar frequencies between the groups (mild, 5 asthmatic vs 3 healthy subjects; moderate, 5 asthmatic vs 5 healthy subjects; and severe, 7 asthmatic vs 8 healthy subjects). 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 asthmatic and healthy groups had similar viral shedding in nasal secretions during the

rhinovirus infection. Both the asthmatic and healthy groups had the highest viral shedding on day 3 (Fig 2, B). There were no significant differences in peak viral titers ($\log_{10} 4.3 \pm 0.8$ vs 3.7 ± 1.4 TCID₅₀/mL). On resolution of the acute infection (2 weeks after inoculation), there was a trend for more asthma subjects to have persistently detectable virus (60% vs 31%, $P = .06$). Peak viral titers were associated with peak symptom scores ($r = 0.45$, $P = .005$) and peak nasal neutrophils ($r = 0.61$, $P < .0001$) but not peak sputum neutrophils ($r = 0.13$, $P = .46$).

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

During the acute cold (day 3), rhinovirus RNA levels in nasal lavage fluid were positively associated with rhinovirus RNA levels in sputum ($n = 25$, $r = 0.59$, $P = .003$). Rhinovirus RNA levels from nasal lavage fluid and sputum were positively associated with nasal lavage fluid quantitative viral cultures ($n = 30$, $r = 0.79$, $P < .0001$ and $n = 29$, $r = 0.55$, $P = .003$, respectively).

Cellularity

In sputum subjects with asthma ($n = 19$; healthy subjects, $n = 15$) were more likely to have detectable eosinophils at baseline (detectable: 56% vs 8%, $P = .008$; 1.2 [25th-75th percentile, 1.0-6.0] vs 0.0 [25th-75th percentile, 0.0-0.9] $\times 10^3$ cells/g), acute infection (82% vs 33%, $P = .04$; 3.0 [25th-75th percentile, 1.3-8.0] vs 0.4 [25th-75th percentile, 0.0-1.3] $\times 10^3$ cells/g), and recovery (72% vs 33%, $P = .14$; 3.2 [25th-75th percentile, 0.9-7.8] vs 0.4 [0.0-1.0] $\times 10^3$ cells/g). In nasal lavage fluid more subjects with asthma had detectable eosinophils at baseline (63% vs 31%, $P = .10$; 0.1 [25th-75th percentile, 0.0-0.3] vs 0.0 [25th-75th percentile, 0.0-0.0] $\times 10^3$ cells/g), acute infection (80% vs 27%, $P = .004$; 0.9 [25th-75th percentile, 0.2-9.2] vs 0.0 [25th-75th percentile, 0.0-0.1] $\times 10^3$ cells/g), and recovery (74% vs 27%, $P = .04$; 0.7 [25th-75th percentile, 0.0-5.7] vs 0.0 [25th-75th percentile, 0.0-0.0] $\times 10^3$ cells/g).

There were minimal group-specific differences in other types of cells in sputum and nasal lavage fluid. In sputum the numbers of neutrophils were similar between groups at baseline (282 [196-380] $\times 10^3$ cells/g for asthmatic subjects vs 166 [116-237] $\times 10^3$ cells/g for healthy subjects), acute infection (384 [144-566] $\times 10^3$ cells/g for asthmatic subjects vs 169 [134-501] $\times 10^3$ cells/g for healthy subjects), and recovery (202 [177-639] $\times 10^3$ cells/g for asthmatic subjects vs 128 [68-214] $\times 10^3$ cells/g for healthy subjects; (asthmatic subjects, $n = 19$; healthy subjects, $n = 15$; see Fig E1, A, in this article's Online Repository at www.jacionline.org). Sputum neutrophil counts increased during the acute cold. No significant changes in either sputum monocyte or lymphocyte counts were noted over the course of the infection (see Fig E1, B and C). In nasal lavage samples neutrophil, monocyte, and lymphocyte counts increased during the acute cold, but there were

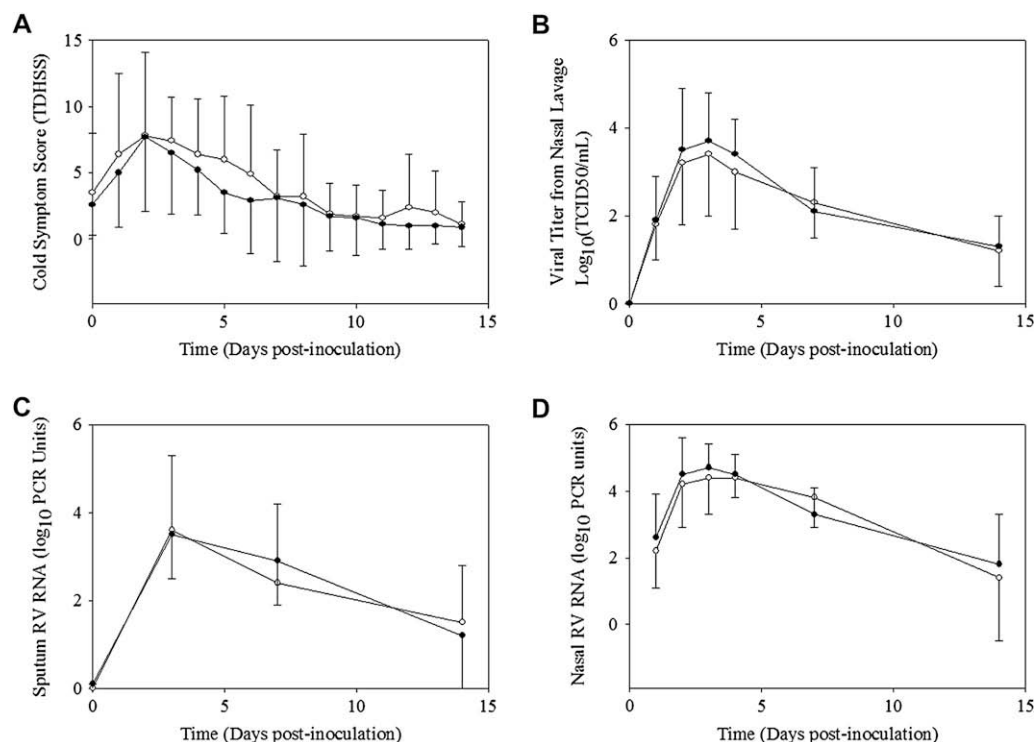


FIG 2. Group-specific outcomes of experimental infection. During the RV16 infection, cold symptom scores (A), nasal lavage fluid viral titers (B), sputum rhinovirus RNA levels (C), and nasal rhinovirus RNA levels (D) were determined in healthy subjects (open circles, $n = 17$) and subjects with allergic asthma (solid circles, $n = 20$). Subjects were inoculated on days 0 and 1. All data are expressed as means $\pm$ SDs. The day 0 sputum values were obtained from samples obtained at the baseline visits.

no differences between the 2 groups (see Fig E2 in this article's Online Repository at www.jacionline.org).

Cytokines in nasal lavage fluid

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 ($P < .001$, see Fig E3 in this article's Online Repository at www.jacionline.org). Peak CXCL8 levels were associated with peak nasal neutrophil counts ($r = 0.67$, $P < .0001$). IFN- $\alpha 2$ and IFN- γ responses were detectable in only about one third of subjects during the acute cold, and levels significantly increased during acute infection compared with baseline values ($P < .001$). Throughout the study, there was no consistent difference between asthmatic and healthy subjects with detectable IFN- $\alpha 2$ or IFN- γ levels. At baseline, more asthmatic than control subjects had detectable CCL2 levels in nasal lavage samples (47% vs 12%, $P = .04$). There were no other group-specific differences in nasal lavage fluid cytokine levels during the course of infection.

Serum CXCL10 and rhinovirus RNA

Serum levels of CXCL10 were assessed during the course of the acute infection. Subjects had similar CXCL10 levels at baseline (210 ± 156 pg/mL for asthmatic subjects vs 152 ± 108 pg/mL for healthy subjects). After inoculation, all subjects had an approximately 3-fold increase in serum CXCL10 levels ($P < .0001$); both groups had a wide range of responses with no group-specific differences ($P = .55$). CXCL10 levels returned to baseline levels

on resolution of the cold (14 days after 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 asthmatic and control subjects at baseline and incubated with RV16 (1×10^7 PFU/mL) to evaluate *ex vivo* cytokine response. Asthmatic ($n = 16$) and control ($n = 16$) subjects had similar cytokine responses, respectively: IFN- $\alpha 2$, 7.0 [25th-75th percentile, 2.6 - 13.9] $\times 10^2$ pg/mL versus 7.6 [25th-75th percentile, 2.6 - 12.2] $\times 10^2$ pg/mL; IFN- γ , 6.6 [25th-75th percentile, 3.9 - 12.1] $\times 10^2$ pg/mL versus 9.6 [25th-75th percentile, 5.5 - 16.3] $\times 10^2$ pg/mL; IL-6, 5.6 [25th-75th percentile, 3.4 - 17.7] $\times 10^2$ pg/mL versus 18.3 [25th-75th percentile, 7.1 - 36.0] $\times 10^2$ pg/mL; and IL-10, 1.1 [25th-75th percentile, 0.6 - 1.6] $\times 10^2$ pg/mL versus 1.1 [25th-75th percentile, 0.5 - 2.1] $\times 10^2$ pg/mL. 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 white blood cells) before inoculation reported more severe cold symptoms ($P = .05$; Fig 3, A). Subgroup analysis indicated that this was true both within the asthmatic and control groups (Fig 3, B). 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 titers (log_{10} TCID₅₀/mL: 4.2 ± 1.1 vs 3.7 ± 1.4 , undetectable vs detectable; $P = .03$), and there

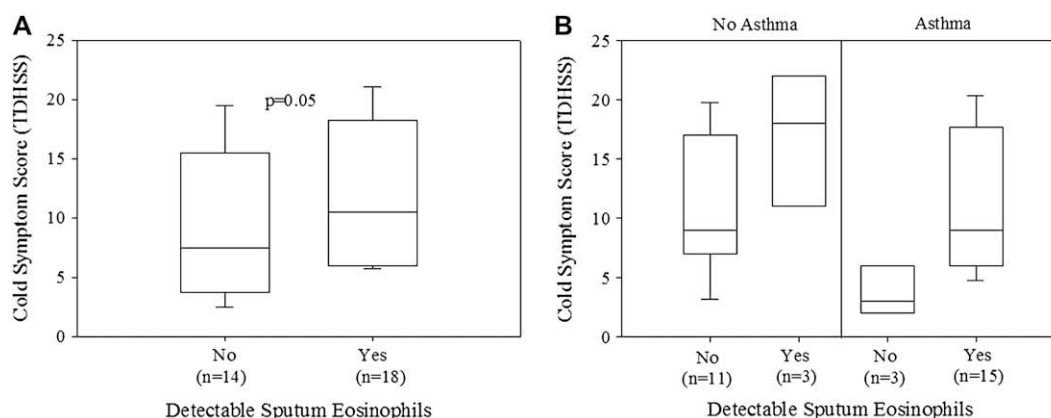


FIG 3. Relationship of sputum eosinophils to cold symptoms. **A**, At baseline, subjects were determined to have detectable (≥ 1 eosinophil per 300 white blood cells in sputum) or undetectable sputum eosinophils. Subjects with detectable sputum eosinophils at baseline had higher peak cold symptom scores with RV16 infection ($P = .05$). 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.

was a trend for an association between baseline CXCL8 levels and peak cold symptom scores (TDHSS: 9.5 ± 5.5 vs 11.9 ± 6.5 , undetectable vs detectable; $P = .08$). 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- $\alpha 2$ secretion and peak cold symptom scores ($r = -0.35$, $P = .07$). Other PBMC cytokine responses (IFN- γ , IL-6, and IL-10) were not related to peak cold symptom scores or peak viral titers.

Total IgE levels were greater in the asthmatic 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, 9 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 titers (3.1 ± 1.6 vs $4.3 \pm 0.8 \log_{10}$ TCID $_{50}$ /mL, detectable vs undetectable; $P = .008$) but was not related to peak symptom score (10.9 ± 6.0 vs $9.2 \pm 5.9 \log_{10}$ TCID $_{50}$ /mL, detectable vs undetectable; $P = .56$).

DISCUSSION

Rhinovirus infections cause greater morbidity in subjects 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 fluid and sputum. RV16 infection caused a modest increase in the peak asthma index only in asthmatic subjects, and none of the subjects with asthma experienced a severe exacerbation. With the exception of increased eosinophil counts in subjects with 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 eosinophil counts and CXCL8 levels in nasal lavage fluid, 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 healthy 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 that asthmatic subjects had greater lower respiratory tract 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 2 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 subjects with more severe asthma 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* might behave differently than cells *in vivo*, perhaps because of 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 after 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 with 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 both asthmatic and control subjects with detectable sputum eosinophils reported more severe symptoms, and this relationship was independent of the effects on viral replication. These findings are intriguing because Rakes et al²⁰ found that rhinovirus infection and airway eosinophilia synergistically increased the risk for wheezing in children. Collectively, these results suggest that eosinophils either contribute to airway inflammation or symptoms in the context of rhinovirus infection. In

accordance with this hypothesis, Davoine et al²¹ recently reported that rhinovirus-induced activation of combined antigen-presenting cells and CD4⁺ T cells could in turn cause eosinophil degranulation. In previous experimental inoculation studies, rhinovirus infections were found to enhance allergen-induced eosinophil recruitment²²; however, it is puzzling that induction of eosinophils by means of allergen challenge before viral inoculation did not increase the severity of the illness.²³ A more recent experimental inoculation study found that greater numbers of eosinophils in bronchoalveolar lavage fluid from asthmatic subjects were significantly related to a larger maximal decrease 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 might have some antiviral activity.²⁴ Additional studies are needed to clarify the relationships, which appear to be complex, between airway eosinophils and rhinovirus-induced illnesses.

Other predictors of respiratory outcomes included baseline levels of airway cytokines and cytokine responses of PBMCs. Baseline CCL2 levels from nasal samples were inversely related to peak viral titer but not symptom score. CCL2 is secreted by rhinovirus-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 PBMCs and peak cold symptom scores ($r = -0.35$, $P = .07$). Unlike previous studies conducted in allergic and healthy subjects without asthma²⁷ and in asthmatic and healthy 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 because of 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 might 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 used 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 3 subjects in the asthmatic group experienced an asthma index of greater than 30, which was defined by Sorkness et al¹⁴ as exceeding the usual variability and the threshold for defining an asthma exacerbation; however, none of them required oral corticosteroids, urgent or emergency department care, or hospitalization indicative of a severe exacerbation. The sample size of this study was sufficient to have 80% power to detect a 1-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 rhinovirus other than RV16 that could have greater differential effects on asthma.

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

Clinical implications: Subjects with mild asthma 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.

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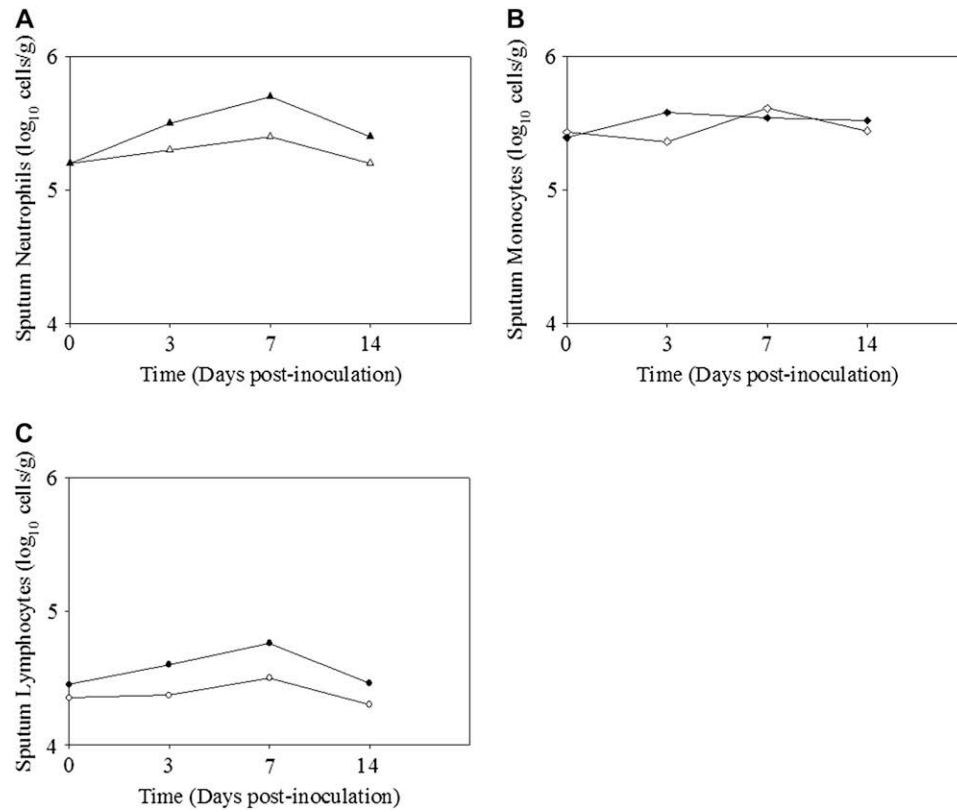


FIG E1. Sputum cellularity for healthy (*open symbols*) and asthmatic (*solid symbols*) subjects after experimental inoculation of neutrophils (**A**; *triangles*), monocytes (**B**; *diamonds*), and lymphocytes (**C**; *circles*). The day 0 sputum values were obtained from samples obtained at the baseline visits.

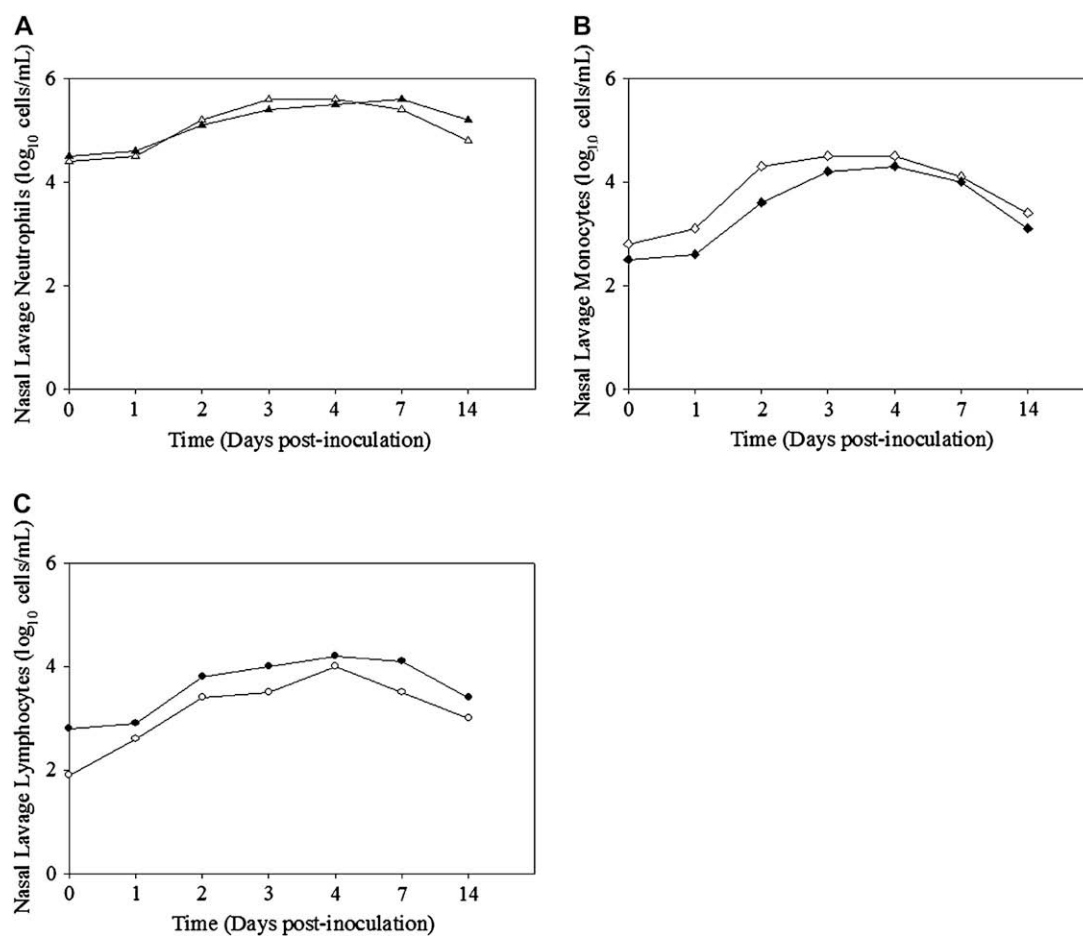


FIG E2. Nasal lavage cellularity for the healthy (*open symbols*) and asthmatic (*solid symbols*) groups after experimental inoculation of neutrophils (**A**; *triangles*), monocytes (**B**; *diamonds*), and lymphocytes (**C**; *circles*).

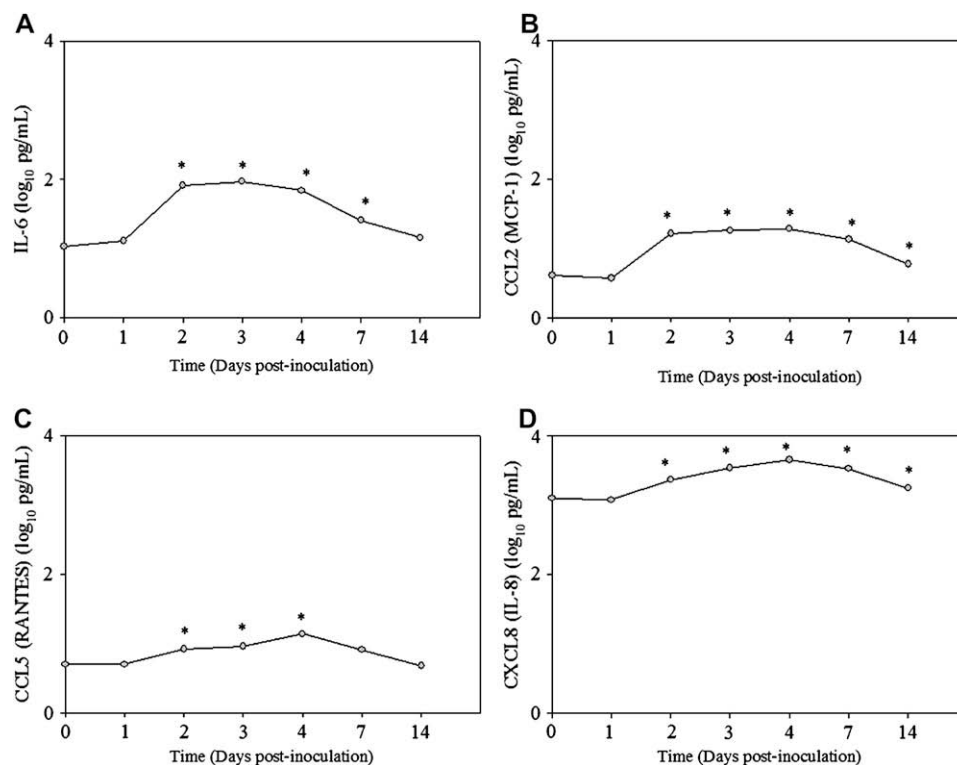


FIG E3. Cytokine levels (median values) from nasal lavage samples of IL-6 (**A**), CCL2 (monocyte chemotactic protein 1 [*MCP-1*]; **B**), CCL5 (RANTES; **C**), and CXCL8 (IL-8; **D**). Asterisks denote a significant increase in median values from baseline ($P < .05$ by using repeated-measures models).