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Impact of community respiratory viral infections in urban children with asthma

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ABSTRACT

Background: Upper respiratory tract viral infections cause asthma exacerbations in children. However, the impact of natural colds on children with asthma in the community, particularly in the high-risk urban environment, is less well defined.

Objective: We hypothesized that children with high-symptom upper respiratory viral infections have reduced airway function and greater respiratory tract inflammation than children with virus-positive low-symptom illnesses or virus-negative upper respiratory tract symptoms.

Methods: We studied 53 children with asthma from Detroit, Michigan, during scheduled surveillance periods and self-reported respiratory illnesses for 1 year. Symptom score, spirometry, fraction of exhaled nitric oxide (FeNO), and nasal aspirate biomarkers, and viral nucleic acid and rhinovirus (RV) copy number were assessed.

Results: Of 658 aspirates collected, 22.9% of surveillance samples and 33.7% of respiratory illnesses were virus-positive. Compared with the virus-negative asymptomatic condition, children with severe colds (symptom score ≥5) showed reduced forced expiratory flow at 25% to 75% of the pulmonary volume ($FEF_{25\%-75\%}$), higher nasal messenger RNA expression of C-X-C motif chemokine ligand (CXCL)-10 and melanoma differentiation-associated protein 5, and higher protein abundance of CXCL8, CXCL10 and C-C motif chemokine ligands (CCL)-2, CCL4, CCL20, and CCL24. Children with mild (symptom score, 1-4) and asymptomatic infections showed normal airway function and fewer biomarker elevations. Virus-negative cold-like illnesses demonstrated increased FeNO, minimal biomarker elevation, and normal airflow. The RV copy number was associated with nasal chemokine levels but not symptom score.

Conclusion: Urban children with asthma with high-symptom respiratory viral infections have reduced $FEF_{25\%-75\%}$ and more elevations of nasal biomarkers than children with mild or symptomatic infections, or virus-negative illnesses.

Introduction

Viral infections are the most common cause of asthma exacerbation in children. Cross-sectional studies of outpatient children who are sick with asthma exacerbations have shown 61% to 81% positivity for viral infection, compared with 21% to 42% of children who are well.[1-4] Rhinovirus (RV) makes up more than 50% of viruses isolated. Viral detection is associated with asthma exacerbation treatment failure.[5]

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Nevertheless, apparently well children with asthma may also harbor respiratory viruses in their airways.[1,6-9] Twenty-one percent of hospitalized children 3 years or older without wheezing tested positive for virus.[3] Similarly, 23% of children 2 years of age or older with well-controlled asthma tested positive for virus.[4] Virus detection rates in healthy children are higher in young children[10-15] and developing communities.[16] Given the high rate of RV transmission within families[17] and the 1- to 3-week duration of RV shedding after infection, most asymptomatic infections likely represent children convalescing from a symptomatic viral infection.[18]

The impact of respiratory viral detection in children with asthma in a community environment is less well studied. In children from Madison, Wisconsin, virus-positive weeks were associated with greater asthma symptoms, as well as more frequent loss of asthma control.[19,20] In a community cohort of children with asthma from Randrick, Australia (a suburb of Sydney), RV was detected in 25.5% of nasal samples and associated with increased cough, phlegm, wheeze, and chest tightness.[21,22] No change in peak expiratory flow (PEF) or forced expiratory volume in 1 second (FEV_1) was seen. However, effects of natural respiratory viral infections on lung function and symptoms in urban children with asthma remain largely undefined. Patterns of viral respiratory illnesses may differ between urban and suburban children. For example, sick inner-city infants have lower rates of viral detection than suburban infants.[23] In addition, asthma is undertreated in urban children,[24] which may amplify the effects of viral infection. Finally, whereas we[25] and others[26-29] have examined nasal cytokine responses of children with asthma to natural colds, potential effects of asymptomatic or mild viral infections have not been studied.

We hypothesize that children with asthma in an urban community environment who experience high-symptom upper respiratory viral infections have reduced pulmonary function as well as greater respiratory tract inflammation and viral copy number than children with virus-positive low-symptom illnesses or virus-negative upper respiratory tract symptoms. We therefore examined the influence of viral infection on respiratory symptoms, lung function, and nasal cytokines in children with asthma from Detroit, Michigan.

Methods

Screening Questionnaire

This study was conducted by Community Action Against Asthma (CAAA), a community-based participatory research partnership, as part of an environmental epidemiology study evaluating the impact of roadway-associated air pollution on asthma health. Children with known or probable asthma living in Detroit, Michigan, were recruited using a screening questionnaire[24] distributed at community venues and through door-to-door recruitment in neighborhoods near highways. The questionnaire asked about demographic information, symptoms, and whether their child had ever been diagnosed by a medical care provider with any of the following conditions: asthma, bronchitis, bronchiolitis, reactive airways disease, or pneumonia. Parents also were asked whether their child had taken prescription medication for these conditions. Classification of asthma severity was based on symptom frequency and reported inhaled steroid use (eTable 1). Children were classified as atopic if they reported having hay fever, nasal allergies, or eczema. This study was approved by the University of Michigan IRB (ID# HUM00018442).

Data and Sample Collection

Fifty-three children participated in a 2-week surveillance assessment period of health status each season from fall 2010 to summer 2011. During each 2-week surveillance period, staff obtained spirometry, symptom reports, and nasal lavage samples during 3 home visits. Respiratory symptoms were assessed by using a modified version of a previously published respiratory symptom score[30] assessing fever, cough, sore throat, nasal symptoms, wheezing, difficulty breathing, and interference with activities (eTable 2). By definition, children with wheezing, difficulty breathing, or breathing fast had symptom scores of 5 or greater. Families were given a calendar and respiratory symptom scale to mark the level of their symptoms.

From winter 2010 to summer 2011, measurements were repeated during a 1-week period whenever the child experienced a symptomatic respiratory illness as defined by a symptom score of 2 or higher (referred to as a "sick period"). We set a low symptom threshold to maximize sensitivity to detect viral illnesses. Families called when the child became ill. When symptoms reached the appropriate threshold, staff would begin a "sick period" assessment within 48 hours of the phone call (median time to first sample was 72 hours after symptom development). Staff also conducted weekly telephone calls to identify illnesses in progress that families may not have reported and initiated a "sick period" collection if the child had current symptoms.

Nasal Lavage

Nasal lavage samples were collected 3 times during a 2-week surveillance period or a 1-week sick period by field staff. Two squirts of isotonic 0.65% NaCl (B.F. Ascher, Lenexa, Kansas) were instilled into the child's nostrils. Subjects then blew their nose into a zippered plastic bag, and 3 mL of M4RT viral transport medium (Remel, Lenexa, Kansas) were added. After collection, samples were placed in transport cooler at 0°C and transported to the Henry Ford Health System Epidemiology Lab for freezing to -70°C, and subsequently transported to Ann Arbor on dry ice.

Detection of Respiratory Viruses

Nasal lavage samples were homogenized (Thermo Fisher Scientific, Waltham, Massachusetts) and nucleic acids extracted using TRIzol-LS (ThermoFisher), chloroform, and an RNeasy Mini Kit (Qiagen, Valencia, California). Samples were analyzed for viral nucleic acid by multiplex polymerase chain reaction (PCR; Seegene Seeplex RV-15 ACE detection kit, Concord, California). This kit detects human adenovirus, bocavirus 1-4, coronaviruses 229E/NL63 and OC43, enterovirus, influenza A and B, metapneumovirus, parainfluenza viruses 1-4, respiratory syncytial virus (RSV) A and B and rhinovirus A, B, and C. For surveillance samples, all specimens were analyzed for virus; for cold samples, specimens from the same sick week were pooled before viral detection analysis (samples from sick periods were not pooled for cytokine or viral copy number determination).

Nasal Lavage Messenger RNA and Protein Expression

All nasal samples were analyzed for messenger RNA (mRNA) and protein. Complementary DNA (cDNA) was synthesized from total RNA by Taqman reverse transcriptase kit (Qiagen). DNA was digested with DNase I (Qiagen). C-X-C motif chemokine ligand (CXCL)-8, CXCL10, interferon regulatory factor 7 (IRF7), retinoic-acid-inducible protein 1 (RIG-I), melanoma differentiation-associated protein 5 (MDA5), Toll-like receptor 3 (TLR3), and interferon (IFN)-λ1 mRNA expression were measured by quantitative polymerase chain reaction (qPCR). Specific primers and probes spanning exon-exon junctions (intron splice-sites) were used to prevent amplification of genomic DNA. Expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using the ΔΔCt method. Reactions with a GAPDH cycle number greater than 35 were not analyzed. CXCL8, CXCL10, CCL2, CCL4, CCL5, CCL20, CCL24, interleukin (IL)-4, IL-13 and ICAM-1 protein levels were determined by multiplex immune

assay (Affymetrix, Santa Clara, California). Biomarkers were chosen based on previous studies showing elevations after RV infection, our interest in examining biomarkers we had not previously studied, difficulty of detecting some biomarkers or cytokines in nasal aspirate fluid, cost, and availability.

RV Copy Number and Typing

For samples testing positive for RV, copy number was determined by qPCR, using previously published primers.[31]

RV Typing

Rhinovirus-positive surveillance samples and 1 sample each from RV-positive sick period were further analyzed to determine RV genotype. Rhinovirus typing was performed by semi-nested PCR amplification of the P1-P2 region from gel-purified PCR products.[32] The identity of each sequence was determined by comparison with known 5′ sequences, using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Spirometry

Using protocols we developed for large-scale community-based asthma studies,[33] staff conducted spirometry to assess lung function during home visits using the EasyOne spirometer (NDD, Andover, Massachusetts).

Fraction of Exhaled Nitric Oxide in Exhaled Breath

Fraction of exhaled nitric oxide in exhaled breath (FeNO) was measured using the NIOX MINO (Aerocrine, New Providence, New Jersey).

Statistical Analysis

Our initial analysis identified that, in addition to anticipated viral-positive illnesses and virus-negative asymptomatic periods, in some surveillance samples viruses were detected and symptomatic illnesses were seen during which no virus was detected. This prompted us to perform a post-hoc analysis looking for similarities and differences between 6 groups: virus-positive/severe symptoms (symptom score ≥5); virus-negative/mild symptoms (symptom score 1-4); virus-negative/asymptomatic (symptom score 0); virus-negative/severe symptoms (symptom score ≥5); virus-positive/mild symptoms (symptom score 1-4); and virus-positive/asymptomatic (symptom score 0). Mean and standard deviation were used to describe nasal biomarker protein levels, nasal mRNA levels and symptom score before and during viral illnesses. Distributions of continuous outcome variables were examined and appropriate transformations taken to achieve normality. Toll-like receptor 3 and IFN-λ1 mRNA levels were undetectable in a large number of samples (eTable 3), and therefore these results were analyzed as a binary variable (detectable, undetectable).

Effects of viral/symptom state on individual symptoms, lung function, nasal aspirate mRNA and protein levels, and RV copy number were determined using generalized estimating equations (GEE), with an exchangeable correlation structure using the identify link for continuous outcomes and the log link for binary ones. Analysis was performed using SAS software (Cary, North Carolina). We evaluated and adjusted for age, sex, ethnicity/race, self-reported atopy, smoker in the home, caregiver educational attainment, season of sample collection, and whether the sample was from a surveillance or sick collection period. Family income, proximity to high-traffic highways, baseline asthma severity, and medication use were evaluated but not included in final models, because they were not significant predictors. Significance level was set at $P < .05$.

Results

Study Participants

Fifty-three children with asthma were enrolled. Surveillance samples were collected from September 2010 to August 2011, and sick samples were collected from December 2010 to August 2011. Subjects were predominantly African-American (Table 1). Most subjects were atopic, exposed to tobacco smoke, and had a household income less than $15,000. Based on symptom frequency and reported inhaled steroid use, most children had mild intermittent or mild persistent asthma. Approximately one-quarter had moderate-to-severe persistent disease and used inhaled corticosteroids within the last year. Of note, this community has a high rate of poorly controlled asthma and undertreatment with inhaled corticosteroids.[24,34] Mean values of forced vital capacity (FVC), FEV_1, and PEF measured at the time of the first surveillance visit were normal, but FEV_1/FVC ratio and forced expiratory flow at 25-75% of the pulmonary volume ($FEF_{25\%-75\%}$) were mildly reduced (Table 2). Average FeNO was elevated. Group mean surveillance nasal aspirate mRNA and protein values are shown in Table 3.

Participant Respiratory Illnesses

From September 2010 to August 2011, 410 surveillance samples were collected, 94 (22.9%) of which were positive for 1 or more viruses. From December 2010 to August 2011, 83 self-reported respiratory illnesses were found, for which 248 samples were collected. Analysis of samples pooled within each individual sick period showed that 28 of 83 (33.7%) of these illnesses were positive for virus. Thus,

Table 1
Participant Baseline Demographic Characteristics (n = 53)

Age, mean (SD)	9.7 (2.1)
Gender, % female	43.4
Race, % Non-Hispanic African-American	86.8
Household income, % ≤$15,000	56.3
Caregiver years of education, %	≤1256.9
Caregiver depression CESD score, mean (SD)	8.8 (5.1)
Smoker in household, % yes	67.9

Table 2
Initial Surveillance Period Health Measures[a]

Asthma severity, N (%)	
Moderate or severe persistent	14 (26.9)
Mild persistent	27 (51.9)
Mild intermittent	11 (21.2)
Atopy (self-reported), % yes	38 (73.1)
Asthma medication use in last 12 months (N, %)	
Inhaled corticosteroids	12 (23.1)
Short-acting bronchodilator only	21 (40.4)
No asthma medication	19 (36.5)
Asthma control test (ACT) score, mean ± SD	20.0 ± 4.2
Symptom score, median (range)	2.3 (0, 27)
Lung function (% of predicted), mean ± SD (range)	
FVC (N = 43)	93.0 ± 16.2 (56.7, 136.7)
FEV_1 (N = 43)	80.3 ± 17.9 (30.2, 125.4)
FEV_1/FVC ratio (N = 43)	75.5 ± 11.9 (38.9, 93.0)
FEF_{25-75} (N = 42)	55.8 ± 20.3 (17.6, 109.3)
PEF (N = 42)	83.7 ± 20.9 (31.3, 122.7)
FeNO (ppb), mean ± SD (N = 50)	26.8 ± 25.7 (6.0, 147.7)

[a]N = 53 except when noted.
Abbreviations: $FEF_{25\%-75\%}$, forced expiratory flow at 25%-75% of the pulmonary volume; FeNO, fraction of exhaled nitric oxide; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; PEF, peak expiratory flow.

Table 3
Participant Viral Infections

Surveillance collection (N = 410)	N	%
No virus	288	70.2 (of total samples)
Virus	94	22.9
Single infections	85	20.7
RV	46	48.9 (of viral infections)
Coronavirus 229E/NL63	9	9.6
RSV A	8	8.5
Coronavirus OC43	5	5.3
RSV B	4	4.3
Influenza A	4	4.3
Influenza B	3	3.2
Adenovirus	2	2.1
Metapneumovirus	2	2.1
PIV2	2	2.1
Multiple infections	9	9.6
Without RV	5	5.3
With RV	4	4.3
Sick collection (N = 248 samples, number of sick periods = 83)		
	No. of sick periods	%
No virus	55	66.3 (of total sick periods)
Virus	28	33.7
Single infections	26	26.1
RV	20	71.4 (of viral infections)
Influenza A	2	7.1
Influenza B	1	3.6
Coronavirus 229E/NL63	1	3.6
PIV 2	1	3.6
RSV B	1	3.6
Multiple infections	2	7.1
Without RV	0	0.0
With RV	2	7.1

Abbreviations: PIV, parainfluenza virus; RSV, respiratory syncytial virus; RV, rhinovirus.

subjects were only slightly more likely to have a virus during self-reported colds than during surveillance sample collection. Rhinovirus was detected in 50 (53.2%) of virus-positive surveillance samples and 22 (78.6%) of virus-positive sick periods (Table 3). Because of the large number of virus-negative self-reported illnesses, we retested the 83 pooled sick period samples for RV, using qPCR. Of the 20 Seegene samples with single RV infections, 17 (85%) were positive for RV by qPCR. Of the 61 Seegene RV-negative samples, 2 (3%) were positive for RV by qPCR. Seventy-four samples from single RV infections were examined for genotyping by qPCR. Sequences of 73 samples showed a specific RV genotype. Median level of identity was 95%. Infections consisted of 60 species A infections, 2 species B infections, and 11 species C infections.

Analysis of Virus-Positive High-Symptom and Low-Symptom Conditions

During surveillance periods, virus-negative samples were associated with a symptom score of 2.6 ± 4.5 (mean ± standard deviation [SD]), and virus-positive samples were associated with a symptom score of 3.1 ± 4.2 ($P = .008$, Wilcoxon rank-sum test). During sick periods, virus-negative samples were associated with a symptom score of 6.4 ± 5.7, and virus-positive samples were associated with a symptom score of 5.7 ± 5.1. Children with virus-positive, severe illnesses (symptom score, ≥5) experienced reductions in $FEF_{25\%-75\%}$ (Fig 1A). Only the symptomatic virus-negative groups demonstrated a significant increase in FeNO (Fig 1B).

Next, we examined nasal aspirate mRNA and protein levels in the 6 conditions. A total of 607 samples were analyzed (Table 4). Of interest, 43 samples were virus-positive and had an associated symptom score of 0 (asymptomatic infection). Of these, 24 had at least 1 sample collected within 28 days before the asymptomatic infection. Eighteen of 24 (75%) were preceded by a cold within that time period, indicating that asymptomatic infections represented convalescence from an earlier symptomatic cold.

Compared with the virus-negative asymptomatic condition, samples from children with more severe colds (symptom score ≥5) showed higher nasal mRNA expression of CXCL10 and MDA5 (Fig 2A) and greater protein abundance of CXCL8, CXCL10, sICAM-1, CCL2, CCL4, CCL20, and CCL24 (Fig 2C). In addition, samples from children

Table 4
Distribution of Samples Based on Viral Detection and Symptom Score

	N	Percent
Virus-positive conditions		
1 = symptom score ≥ 5	55	32.4
2 = symptom score 1-4	72	42.3
3 = symptom score 0	43	25.3
Virus-negative conditions		
4 = symptom score ≥ 5	130	21.42
5 = symptom score 1-4	136	22.41
6 = symptom score 0	171	28.17

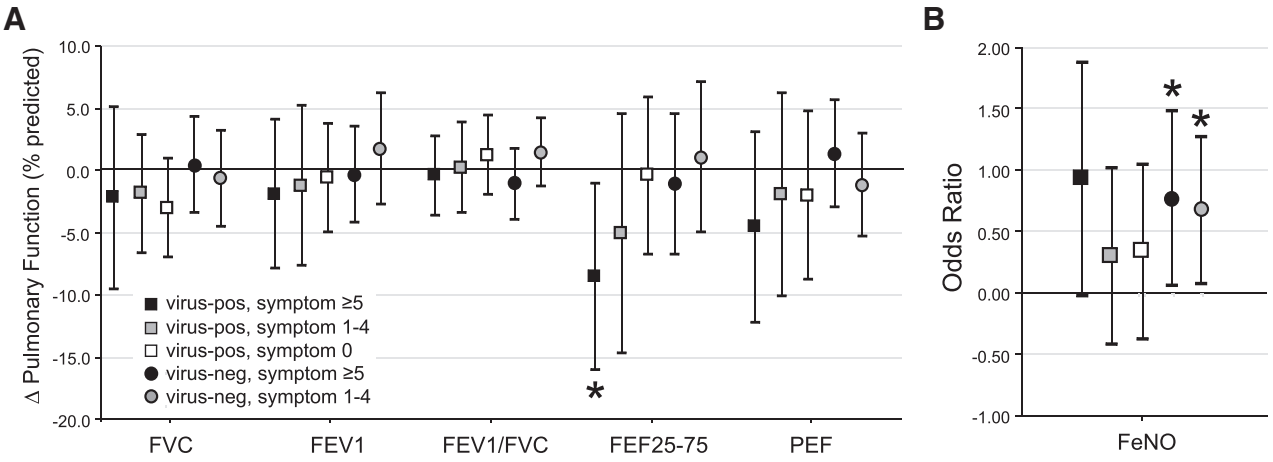

Figure 1. Comparisons of lung function and exhaled NO (eNO) between the 6 groups of conditions. The virus-negative/high-symptom group (symptom score ≥5, black squares), virus-positive/mild-symptom group (symptom score 1-4, gray squares), virus-positive/asymptomatic (symptom score 0, white squares), virus-negative/high-symptom group (symptom score ≥5, black circles) and virus-negative/mild-symptom group (symptom score 1-4, gray circles) are each compared with the virus-negative/asymptomatic group. A, Changes in lung function (percent predicted) compared with the virus-negative/low-symptom group. Adjusted mean estimates and 95% confidence intervals are shown. B, Changes in eNO (ppb) compared with the virus-negative/no-symptom group. Adjusted odds ratios and 95% confidence intervals are shown.

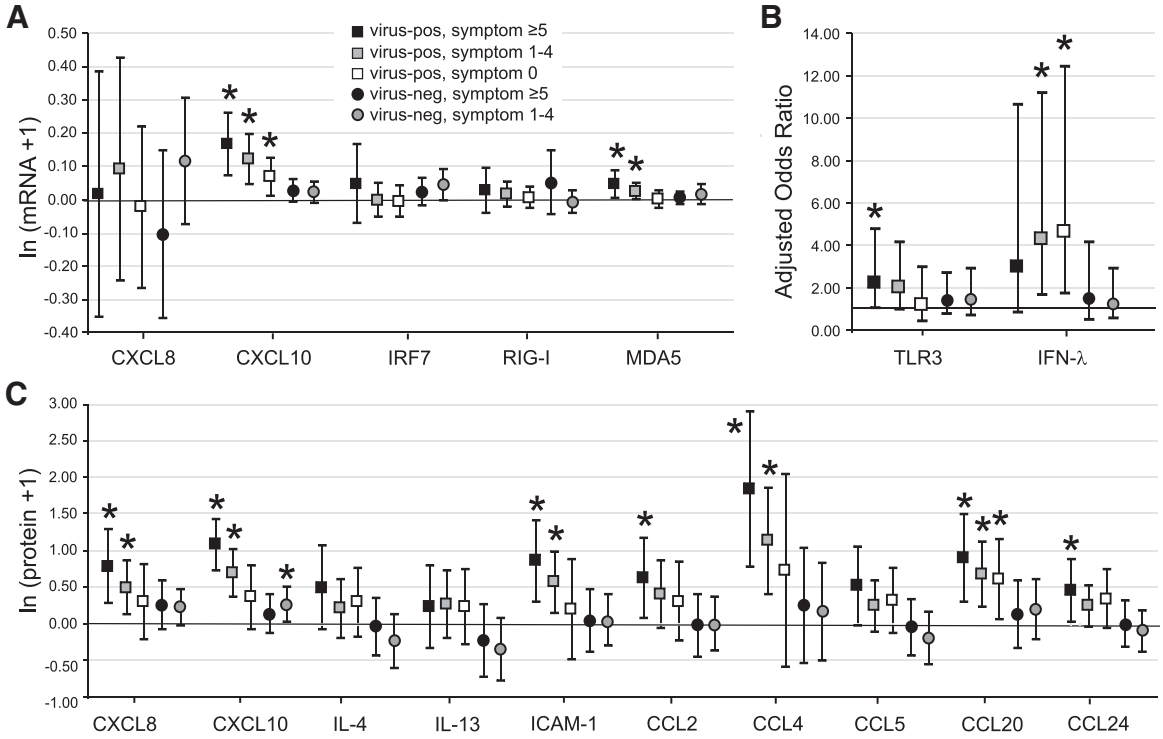

Figure 2. Comparison of nasal aspirate mRNAs and proteins between the 6 groups of conditions. The virus-negative/high-symptom group (symptom score ≥5, black squares), virus-positive/mild-symptom group (symptom score 1-4, gray squares), virus-positive/asymptomatic (symptom score 0, white squares), virus-negative/high-symptom group (symptom score ≥5, black circles), and virus-negative/mild-symptom group (symptom score 1-4, gray circles) are each compared with the virus-negative/asymptomatic group. A, Differences in log transformed mean mRNA values for CXCL8, CXCL10, IRF7, RIG-I, and MDA5 compared with the virus-negative/low-symptom group. The 95% confidence intervals are also shown (*$P < .05$). B, TLR3 and IFN-λ1 mRNAs were analyzed as a binary variable (detectable, undetectable). Odds ratios and 95% confidence intervals compared with the virus-negative/low-symptom group are shown (*$P < .05$). C, Differences in log-transformed mean mRNA values for CXCL8, CXCL10, IL-4, IL-13, sICAM-1, CCL2, CCL4, CCL5, CCL20, and CCL24 compared with the virus-negative/low-symptom group. The 95% confidence intervals are also shown (*$P < .05$). Abbreviations: CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; IFN-λ1, interferon λ1; IL, interleukin; IRF7, interferon regulatory factor-7; MDA5, melanoma differentiation-associated protein 5; mRNA, messenger RNA; RIG-I, retinoic-acid-inducible protein 1; sICAM, soluble intercellular adhesion molecule; TLR3, Toll-like receptor 3.

with more severe colds were more likely to have detectable levels of TLR3 mRNA (Fig 2B). Samples from children with mild and asymptomatic viral infections also showed significant increases in some nasal aspirate biomarkers, albeit fewer biomarkers than samples from more severe colds (Fig 2A, 2C). Samples from children with asymptomatic infections showed higher nasal CXCL10 mRNA and CCL20 protein expression, suggesting persistence of these cytokines after resolution of symptoms. Children with mild colds and asymptomatic infections, but not those with severe colds, also showed an increase in the number of aspirates positive for IFN-λ mRNA (Fig 2B). Finally, and unexpectedly, symptomatic virus-negative illnesses showed no significant increases in nasal biomarkers except for CXCL10 protein. When we re-examined data, restricting our analysis to children with persistent asthma (either mild or moderate-to-severe), nearly identical results were obtained, except that samples from all 3 virus-positive groups showed significant increases in IFN-λ compared with the virus-negative, asymptomatic condition (not shown).

The considerable number of virus-negative illnesses led us to examine the distribution of subjects with self-reported atopy and proximity to highways in the 6 viral detection/symptom score conditions. However, no difference was seen in the percentage of children with atopy (χ^2, 0.24) or high-traffic exposure (0.46) in the virus-negative illness groups. Also, when we reevaluated our data for effect modification using interaction modeling, interaction terms for atopy and high traffic were not statistically significant. Finally, when we reran our GEE models adjusting for the main effects of atopy or high traffic, no change was seen in the associations of interest between virus/symptom group and cytokine level.

Relationships of RV Copy Number to Nasal Aspirate Biomarkers and Respiratory Symptoms

For RV infections, we examined the association between viral copy number with nasal aspirate mRNA, protein, and overall symptom scores. Nasal aspirate RV copy number was positively associated with mRNA expression of CXCL10 and MDA5 (Fig 3A). Viral load also was associated with protein abundance of CXCL8, CXCL10, ICAM-1, CCL2, CCL4, and CCL20 (Fig. 3B, 3C), which together attract neutrophils and monocytes to the airways. However, levels of IL-4, IL-13, CCL5, and CCL24, which promote eosinophil chemotaxis and allergic airways disease, were not associated with viral load. No significant difference was found in viral copy number between symptomatic and asymptomatic infections (symptomatic, $1.22 \times 10^5 \pm 125$ copies/mL; asymptomatic, $0.54 \times 10^5 \pm 54$ copies/mL; geometric mean ± geometric SD, $P = .62$, Wilcoxon rank sum test). Nor was an association seen between viral copy number and overall symptom score ($P = .42$). The study did not have statistical power to correlate copy number with lung function.

Time Course of Viral-Induced Cytokine Expression

Although we did not design the study to examine the time course of cytokine expression, our collections included 3 samples from each of 28 sick period single viral infections, as well as baseline surveillance samples for these individuals. Messenger RNA expression of CXCL10, IRF7, and MDA5 and protein abundance of CXCL10 peaked 1 to 3 days after the onset of infection (Fig 4A). Protein abundance of CXCL8, CXCL10, CCL4, CCL20, and ICAM1 peaked at 4 to 6 days

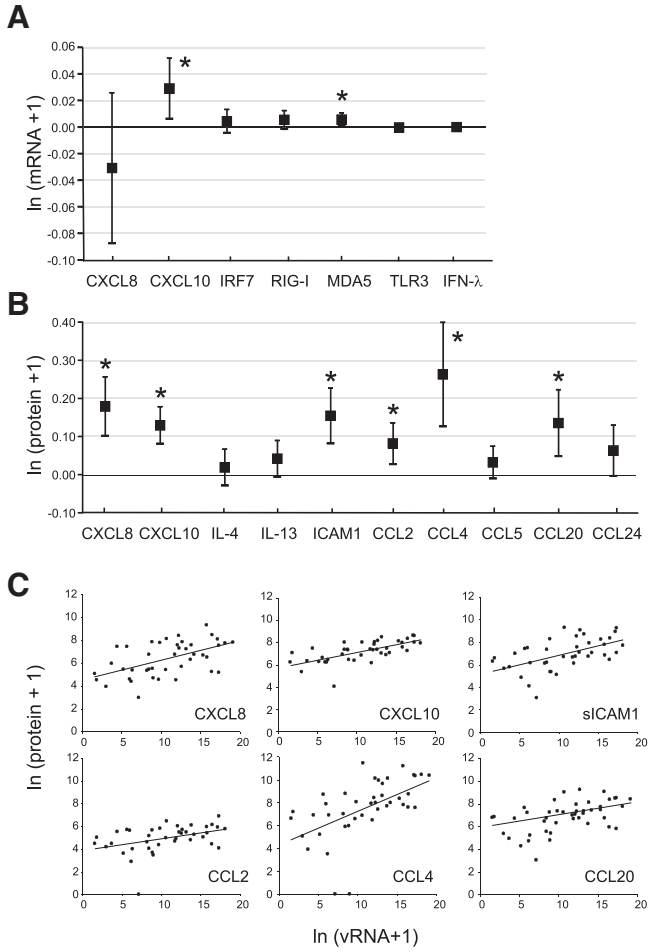

Figure 3. Associations between nasal aspirate biomarkers and rhinoviral RNA. A, Nasal aspirate mRNAs are represented as ln (mRNA +1). Adjusted means and 95% confidence intervals are shown. B, Nasal aspirate proteins represented as ln (protein level +1). Adjusted means and 95% confidence intervals are shown. The association of viral copy number and nasal biomarker was determined using the GEE method (*$P < .05$). C-G, Individual adjusted correlations of viral copy number and selected nasal aspirate cytokines. Abbreviations: GEE, generalized estimating equations; mRNA, messenger RNA.

(Fig 4B). Interleukin-4, IL-13, CCL5, CCL24, each of which promote type 2 inflammation, peaked 7 to 14 days after infection.

Discussion

This study was undertaken to examine the influence of natural upper respiratory tract viral infection on respiratory symptoms, airway function, and inflammation in children with asthma from the urban community, and to determine possible mechanisms by correlating these outcomes with nasal aspirate cytokines and other biomarkers. We hypothesized that children with high-symptom severe colds have greater airflow obstruction, respiratory tract inflammation, and viral copy number than those with milder colds and asymptomatic infections, as well as virus-negative conditions. In contrast to children from a suburban setting,[22] high-symptom viral infections were associated with reduced small airway function, as evidenced by changes in $FEF_{25\%-75\%}$. Compared with the virus-negative asymptomatic condition, children with severe colds showed elevations of nasal mRNA and protein biomarkers. Children with mild and asymptomatic infections showed fewer elevations. We found associations between viral load and nasal aspirate levels of chemokines, which together attract neutrophils and monocytes to the airways, but not those that promote eosinophil chemotaxis and allergic airways disease. This is the first study to correlate viral load and respiratory tract cytokine levels during natural colds. Finally, urban children with asthma experienced many virus-negative symptomatic illnesses, which were associated with increased exhaled nitric oxide but not reduced airway function or elevated nasal biomarkers.

We found that subjects were only slightly more likely to have a virus during self-reported colds (33.7%) than during surveillance sample collection (22.9%). The high rate of viral detection during surveillance periods is consistent with previous studies in well children with asthma[1-4] and is unlikely to be attributable to false positives, because RV detection was confirmed by amplification and sequencing of gel-purified PCR products in 92% of cases. Conversely, we believe the low viral detection rate during symptomatic episodes can be explained by the fact that samples were not collected in the fall and instead were only collected from January to August, when rhinovirus infections are less prevalent. The low symptom threshold for "sick" sample collection and financial reimbursement for each sick period assessment, which offset time and effort needed to participate, could also have contributed to a low viral detection rate. While our rate of viral detection is lower than reported previously,[1,2] it is consistent with the lower frequency of viral detection in urban children with respiratory illnesses compared with suburban children.[23] Finally, our data indicate that urban children with asthma experience frequent virus-negative upper respiratory tract illnesses. Cold-like illnesses were unlikely to represent false-negative viral infections, because they were unaccompanied by reduced pulmonary function or increases in nasal aspirate MDA5, a double-stranded RNA pattern recognition receptor that was increased in virus-positive samples and has been shown to be induced after RV infection.[35] Finally, when we retested a subset of our initial multiplex PCR viral detection results using standard RV qPCR, we found a false-negative rate of only 3%. As in previous studies,[23,30] RV was the most common virus detected, and overall, inner-city infants had low rates of viral detection. However, we did not appreciate higher adenovirus rates as observed previously.[23] Although RV-C is associated with severe asthma exacerbations,[32,36-42] we did not recruit enough patients to discern a difference in symptom severity between species.

Next, we evaluated the significance of viral infection on respiratory symptoms, lung function, and respiratory tract inflammation. One third of viral infections were associated with severe colds (symptom score ≥5), which by definition included children with wheezing, difficulty breathing, or breathing fast. In contrast to children with asthma from a suburb of Sydney,[22] children from Detroit with severe colds demonstrated significant reductions in $FEF_{25\%-75\%}$, consistent with small airways involvement.[43] This discrepancy could relate to the frequent undertreatment of urban children with asthma.[24] Because inhaled corticosteroid use reduces the rate of exacerbations,[44] urban children may be more susceptible to viral-induced reductions in airway function than suburban children. This hypothesis is consistent with previous studies in which the impact of pollution on asthma was seen predominantly in children not using steroids.[45,46]

In addition, we found that high-symptom severe viral infections, but not virus-negative illnesses, were associated with significant elevations in nasal aspirate mRNA expression of CXCL10, MDA5, and TLR3 and protein abundance of CXCL8, CXCL10, sICAM-1, CCL2, CCL4, CCL20, and CCL24. Previous studies of children with natural colds have shown increases in nasal chemokines.[25-29] Increases in MDA5, TLR3, and ICAM-1 are noteworthy, because each functions as a receptor for RV. Melanoma differentiation-associated protein 5 and TLR3 are cytoplasmic and endosomal receptors for viral double-stranded RNA, respectively,[47] whereas ICAM-1 is a receptor for major group RV.[48] Samples from children with mild and asymptomatic viral infections also showed significant increases in nasal aspirate biomarkers, albeit fewer than samples from more severe colds. One fourth of

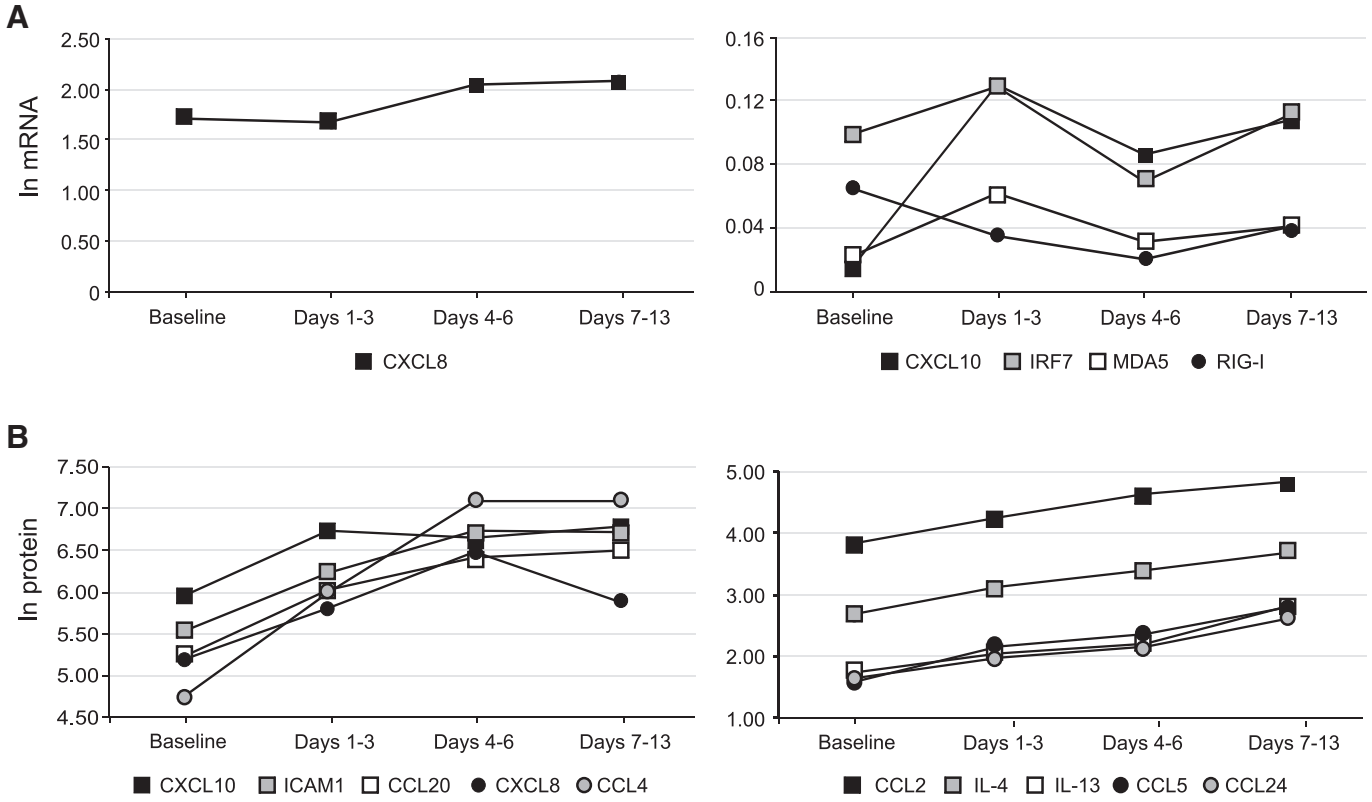

Figure 4. The time course of nasal biomarker changes from 28 virus-positive sick period weeks. A, Nasal aspirate mRNAs are represented as ln mRNA. B, Nasal aspirate proteins are represented as ln protein. For easier readability, SD are not shown. Abbreviations: mRNA, messenger RNA; SD, standard deviation.

virus-positive samples were not associated with symptoms. Collection of these samples often followed symptomatic viral infections, indicating that asymptomatic infections represented convalescence from more severe colds. Samples from children with asymptomatic infections showed higher nasal CXCL10 mRNA and CCL20 protein expression, suggesting persistence of these cytokines after resolution of symptoms.

We hypothesized that viral copy number determines asthma symptoms and respiratory tract inflammation after RV infection. We found that viral load was associated with protein abundance of CXCL8, CXCL10, CCL2, CCL4, and CCL20, chemokines that together attract neutrophils and monocytes to the airways, but not with IL-4, IL-13, CCL5, or CCL24, cytokines that promote eosinophil chemotaxis and allergic airways disease. In contrast, no association was seen between viral copy number and symptoms. Our data are consistent with a previous study in adults with asthma showing no correlation between severity of lower respiratory tract symptoms and viral load.[49] These data suggest that respiratory symptoms in children with asthma with natural colds may not depend on viral load alone. Other factors may determine asthma control after viral infection, including environmental and genetic factors.[50]

The exact nature of the observed virus-negative respiratory tract illnesses is unclear. Unlike virus-positive illnesses, virus-negative symptomatic sicknesses were accompanied by increased eNO. Previous studies in subjects with asthma have shown that experimental exposure to allergen and particulate matter increases eNO in contrast to experimental RV infection, which does not,[51,52] suggesting that virus-negative illnesses could have been precipitated by environmental exposures. We did not find an association between viral-negative illnesses and self-reported atopy or proximity to high-traffic roadways. However, because the 6 groups we studied do not represent different subjects but different disease states according to viral detection and symptoms, possibly these illnesses represent acute exposures to allergen, traffic, or other pollutants.

African American children living in low-socioeconomic-status urban environments continue to experience higher asthma morbidity than white children.[53] Racial disparities are observed in asthma prevalence, emergency department visits, hospital readmissions, and death rates.[54,55] Therefore, although data from Detroit may not be generalizable to other settings, they provide new insight into the effect of viral infections on an important pediatric population.

Our study has a number of limitations. First, we used nasal aspirates to sample respiratory tract inflammation, allowing repeated collection of samples from children in a noninvasive manner. We did not validate our method by comparing our results with lower respiratory tract specimens. However, gene expression among children with asthma is altered similarly in nasal and bronchial airways.[56] Second, a lag period occurred between onset of respiratory symptoms and nasal aspirate collection. Third, symptoms may have been caused or prevented by any number of unmeasured covariates, including body mass index, mucus production, or anti-inflammatory cytokines. Fourth, our assessments of symptom score, asthma severity, and atopy were based on self-report and were not independently validated, allowing the possibility of measurement error.

We conclude that, in urban children with chronic asthma, high-symptom respiratory viral infections reduce airway function. Children with more severe colds demonstrate more elevations of nasal biomarkers than children with mild colds, asymptomatic infections, or virus-negative illnesses. However, many children experienced virus-negative cold-like illnesses associated with increased eNO but not nasal aspirate biomarkers or lung function change. Further studies are needed to understand the precise factors that determine respiratory tract symptoms in children with asthma.

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Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.anai.2018.10.021.

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eTable 1
Classification of Asthma and Asthma Severity Based on Symptom Frequency and Reported Inhaled Steroid Use[a]

I. A child will be considered to have **probable asthma** (of any severity) if any of the following are true:
- a) 3 or more of the 6 non–exercise-related symptoms (ie, questions 3, 4, 5, 6, 9, and 10) were reported (at any level of frequency greater than "never"):
  - Q3. In the past 12 months, how often, on average, has your child seemed congested in the chest or coughed up phlegm (mucus) when he/she did not have a cold or the flu?
  - Q4. In the past 12 months, has your child had wheezing or whistling in the chest when he/she had a cold or the flu?
  - Q5. In the past 12 months, how often, on average, has your child had wheezing or whistling in the chest when he/she did not have a cold or the flu?
  - Q6. In the past 12 months, has your child's wheezing or whistling in the chest ever been severe enough to limit your child's speech to only 1 or 2 words at a time between breaths?
  - Q9. In the past 12 months, how often, on average, did your child wake up from sleep because of wheezing, dry cough, tightness of the chest, or shortness of breath?
  - Q10. In the past 12 months, how often, how many days (or part of days) of school has your child missed because of wheezing or asthma?
- b) Either exercise symptom (ie, questions 7 and 8) was reported with a frequency of 3 times or more in the past year:
  - Q7. In the past 12 months, how often, on average, has your child sounded wheezy during or after exercise, running, or playing hard?
  - Q8. In the past 12 months, how often, on average, has your child coughed during or after exercise, running, or playing hard?
- c) There is a diagnosis of asthma (ie, yes on question 13) with any symptoms (questions 3 through 10) or doctor-prescribed medication use (ie, yes on questions 14 and 15):
  - Q13. Has any doctor, nurse, or other health professional ever said that your child has asthma, reactive airway disease, asthmatic bronchitis, or wheezy bronchitis?
  - Q14. In the past 12 months, has your child taken any medications, inhalers (puffers), or nebulizers (breathing treatments) prescribed by a doctor for any of the conditions just mentioned?
  - Q15. Does your child take any doctor-prescribed medications for a breathing problem every day, even when he or she is not having trouble breathing?

II. A child will be considered to have **probable moderate to severe asthma** if, first, the child meets the diagnostic criteria for asthma above, and, second, any of the following are true:
- a) any daytime symptom (ie, questions 3 through 9) is reported as being present "every day"
- b) sleep disturbance (question 10) is reported "more than *2 times* per week" or "most nights"
- c) daily use of doctor-prescribed medication use (ie, yes on questions 14 and 15)

III. A child will be considered to have **probable mild persistent asthma** (of any severity) if, first, the child meets the diagnostic criteria for asthma above, second, the criteria for probable or known moderate to severe asthma are **not** met, and, thirdly, any of the following are true:
- a) 1 or more daytime symptoms are reported as being present "more than 2 times per week"
- b) sleep disturbance reported is reported "more than *1 time* per month"
- c) daily use of doctor-prescribed medication use (ie, yes on questions 14 and 15)

IV. A child will be considered to have **probable mild intermittent asthma** if, first, the child meets the diagnostic criteria for asthma above, and, second, neither the criteria for probable or known moderate to severe asthma nor the criteria for probable or known mild persistent asthma are met.

[a]Relevant questions from the screening questionnaire are shown.

eTable 2
Respiratory Symptom Score[a]

	Mild	Moderate	Severe
Fever:	(1)		
Cough:	(1)	(2)	(3)
Runny nose:	(1)	(2)	
Stuffy nose:	(1)	(2)	
Sore throat:	(1)		
Duration of illness >4 days	(1)		
Wheezing:	(5)		
Difficulty breathing:	(5)		
Breathing fast:	(5)		
NOT going to school OR			
NOT doing usual activities:	(5)		

[a]Total score was the sum of all reported components.

eTable 3
Initial Surveillance Period Nasal Biomarker Levels

mRNA[a]	N (total)	N (% detectable)[b]	Median	IQR	(Min-Max)
CXCL8	123	96 (78%)	5.28	(2.9-10.6)	(0-94.35)
CXCL10	123	96 (78%)	0.005	(0-0.019)	(0-3.84)
IRF7	123	96 (78%)	0.05	(0.02-0.11)	(0-0.58)
RIG-I	123	96 (78%)	0.01	(0-0.03)	(0-60.97)
MDA5	123	96 (78%)	0.01	(0-0.03)	(0-1.39)
TLR3	123	56 (45.5%)		(0-1)	
IFNλ1	123	25 (20.3%)		(0-1)	

Protein (pg/mL)[c]	N (total)	Median	IQR	(Min-Max)
CXCL8	136	128.05	(62.2-359.4)	(5.4-3763.5)
CXCL10	134	478.05	(264.3-672.9)	(21.6-3176.2)
IL-4	139	15.3	(3.3-71.6)	(0-428.9)
IL-13	133	0.00	(0-41.9)	(0-254.6)
sICAM-1	135	366.1	(104.7-802.1)	(0-3341.7)
CCL2	137	74.7	(20.4-145.8)	(0-452)
CCL4	135	459	(32.1-1683.9)	(0-32230)
CCL5	137	4.4	(0-19.8)	(0-121.8)
CCL20	140	390	(76.9-810.8)	(0-8493.3)
CCL24	139	5.37	(0-20.5)	(0-73.3)

[a]Normalized to GAPDH.
[b]Indicates number of samples with detectable signal at cycle number ≤35.
[c]Lower limits of detection: CXCL8, 1.2 pg/mL; CXCL10, 0.3 pg/mL; IL-4, 1.5 pg/mL; IL-13, 0.1 pg/mL; sICAM-1, 2.72 pg/mL; CCL2, 0.6 pg/mL; CCL4, 4.7 pg/mL; CCL5, 0.2 pg/mL; CCL20, 2.0 pg/mL; CCL24, 0.34 pg/mL.
Abbreviations: CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN. interferon; IL, interleukin; IRF7, interferon regulatory factor-7; MDA5, melanoma differentiation-associated protein 5; RIG-I, retinoic-acid-inducible protein 1; sICAM, soluble intercellular adhesion molecule; TLR3, Toll-like receptor 3.