

Human rhinovirus in bronchial epithelium of infants with recurrent respiratory symptoms

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Background: Human rhinoviruses (HRVs) are a common cause of upper respiratory tract infections. There is growing evidence that HRVs are also important in lower respiratory tract infections and often induce asthma exacerbations.

Objective: We evaluated the presence of HRV in the lower respiratory tract by obtaining bronchial biopsies from infants with recurrent asthmalike respiratory symptoms.

Methods: A total of 201 steroid-naïve infants age 3 to 26 months with recurrent respiratory symptoms for at least 4 weeks within the preceding 2 months were studied for lung function using body plethysmography. Bronchoscopy was performed in 68 children, and bronchial biopsies were available from 59 infants for HRV detection with *in situ* hybridization.

Results: Human rhinovirus was detected in 21 of 47 (45%) specimens. Abnormal lung function (decreased airways conductance) was found in 18 of 21 (86%) HRV⁺ infants and in 15 of 26 (58%) HRV⁻ infants ($P = .037$). Occurrence of a respiratory infection in the 6 weeks preceding bronchoscopy correlated with HRV positivity ($P = .036$).

Conclusion: Human rhinovirus is frequently found in the lower airways in infants with recurrent respiratory symptoms, and the majority of these HRV⁺ infants also showed increased airway resistance.

Clinical implications: Human rhinovirus is a common pathogen causing upper and lower respiratory symptoms. Follow-up of these infants will reveal whether the presence of HRV in the bronchial biopsy and abnormal lung function with recurrent respiratory symptoms predicts subsequent asthma. (J Allergy Clin Immunol 2006;118:591-6.)

Key words: Asthma, infant, lung function, pediatric, rhinovirus

Human rhinoviruses (HRVs) are the most common cause of upper respiratory infections in children and adults,¹ and almost all children have at least 1 HRV infection by the age of 2 years.² The use of sensitive viral diagnostic tests, based on PCR, has established the importance of viruses in causing lower respiratory infections. The total viral detection rate is approximately 80% among hospitalized wheezing children under 3 years of age, and HRVs have been detected in 20% to 41% of this infant population.³⁻⁶ The importance of HRV causing lower respiratory infections increases with age, and hence, HRVs appear to be the most important viruses associated with exacerbations of asthma⁷ and wheezing requiring hospitalization in older children.⁶ In a recent article by Lemanske et al,⁸ HRV illness during infancy was shown to predict subsequent childhood wheezing.

Studies with *in situ* hybridization (ISH) and PCR have shown that HRVs reach the lower respiratory tract and replicate in the epithelial cells, indicating that a positive viral finding is not caused by a contamination from the upper airways.⁹⁻¹³ Although temperature of $\leq 35^{\circ}\text{C}$ in the upper airways is optimal for HRV replication, the temperature in the proximal lower airways is also cooler than the body core temperature, thus enabling HRV replication.^{14,15}

Host factors such as atopy, decreased lung function, and bronchial hyperreactivity may contribute to increased lower airway symptoms and morbidity of HRV infection.¹⁶ Virus-induced and allergen-induced inflammation may amplify each other.^{6,17} In a study by Kotaniemi-Syrjänen et al,⁴ hospitalization for HRV-induced wheeze in infancy was associated with early school-age asthma. Interestingly, HRV infections were associated with the presence of atopic dermatitis in infancy. There are also findings confirming that an aberrant immune response to HRV may be involved in the development of acute exacerbations in atopic subjects with asthma.¹⁸⁻²⁰

We have earlier shown that an eosinophilic inflammation was not present in endobronchial biopsies in infants with recurrent respiratory symptoms and reversible airway obstruction measured by infant body plethysmography.²¹ The numbers of all inflammatory cells were low relative to the number of structural cells. In the current study, we used ISH to detect HRV from the lower respiratory

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Abbreviations used

ECP: Eosinophil cationic protein
 FRC: Forced respiratory capacity
 HRV: Human rhinovirus
 ISH: In situ hybridization
 nt: Nucleotide
 sGaw: Specific airway conductance
 SPT: Skin prick test

tract—that is, from endobronchial biopsies from the group of infants with recurrent respiratory symptoms. Moreover, the lung function and markers of atopy were defined, and the findings were correlated with the HRV-ISH results.

METHODS**Subjects**

Full-term infants (>36 weeks gestation) age 3 to 26 months with recurrent respiratory symptoms including dyspnea, cough, and/or wheeze for at least 4 weeks within the 8 weeks preceding their first visit were included. Exclusion criteria were use of inhaled corticosteroids within the 8 weeks preceding their lung function visit, a cumulative lifetime systemic corticosteroid use of more than 3 days at a dose of 2 mg/kg or inhaled corticosteroids for more than 4 weeks, nonwhite ethnicity, small for gestational age (birth weight <–2 SD), height at the time of investigation <–2.5 SD, respiratory distress syndrome, bronchopulmonary dysplasia, respiratory infection in the 14 days preceding lung function measurement, severe atopic dermatitis (>20% of body area at the first visit), obvious bronchomalacia or other structural defect, and specific airway conductance (sGaw) <35% of predicted value combined with dyspnea at rest.²¹ Blood samples were drawn for analysis of total eosinophils, total IgE, and eosinophilic cationic protein (ECP).

The study was approved by the Ethics Committee at the Helsinki University Central Hospital, and informed consent was obtained from parents before the study.

Study design

Infants had been referred to a tertiary pediatric center at the Skin and Allergy Hospital, Helsinki University Central Hospital, for investigation of recurrent respiratory symptoms during the period from October 2000 to September 2003. As part of their clinical assessment, the infants underwent lung function tests, and the infants with more severe, prolonged, or atypical symptoms also underwent bronchoscopy that was performed to exclude structural airway abnormalities such as laryngomalacia, tracheomalacia, or bronchomalacia, subglottis stenosis, and other diagnoses such as foreign body inhalation or mucus plugging.²¹ Symptoms of respiratory infection were recorded in the symptom diary for a period of 6 weeks before and as long as 6 weeks after the bronchoscopy by the parents.

Lung function

Functional residual capacity (FRC) and sGaw were measured with an infant whole-body plethysmograph (BabyBody, Jaeger GmbH, Wurtzburg, Germany).^{21,22} The infants inhaled 0.6 mg salbutamol via a steel spacer, Nebuchamber (AstraZeneca, Lund, Sweden), and a positive bronchodilator reversibility was considered if sGaw had decreased >30% after 15 minutes. Paired sets of measurements were performed to assess mean baseline FRC and sGaw and their individual between-test variability. The results were expressed as *z*

scores, which equal the number of SDs by which the observed value deviates from the height-adjusted predicted value.²³ FRC higher than that of the 95th centile (*z* score > +1.65) and sGaw lower than that of the 5th centile (*z* score < –1.65) of the reference range were considered abnormal.

Atopy

Atopy was tested with a skin prick test (SPT) and defined as at least 1 wheal ≥ 3 mm in diameter to the tested allergens in the absence of a response to the negative control solution. SPT were performed for birch, timothy, cat, dog, *Dermatophagoides pteronyssinus*, latex, hen's egg, and cow's milk. Atopic eczema was also considered as a marker of atopy.

Bronchoscopy

Bronchoscopy with endobronchial biopsy was performed through the mouth in the infants under general anesthesia, with a size 3.5-mm rigid bronchoscope, using biopsy forceps (#10378L; Karl Storz, GmbH & Co, Tuttingen, Germany). One or 2 biopsies were taken from the main carina.

Rhinovirus diagnostics

The biopsy specimens were fixed in formaldehyde, dehydrated, and embedded in paraffin. The specimens were cut into 5-μm-thin preparations on Super Frost Slides (Menzel GmbH, Braunschweig, Germany). Preparation of the rhinovirus-14 probe (antisense and sense) and the ISH protocol were based on published procedures.²⁴ The rhinovirus probe used represents a region (nucleotide [nt] 332–572) of the RV14 5′-untranslated region. The probe was amplified by PCR from a cDNA template and cloned into pGEM-4Z plasmid (Promega, Madison, Wis) between HindIII and XbaI sites. The 240-nt fragment was cloned in both orientations under the control of T7 promoter. Digoxigenin-labeled RNA probes were synthesized by using T7 RNA polymerase and the DIG RNA labeling kit (Roche Molecular Biochemicals, Espoo, Finland). ISH was performed by using an automated Ventana Gen II ISH (Ventana Medical Systems, Inc, Tucson, Ariz) using the Ventana Regular protocol. The probe was detected with the Ventana Basic DAB (3,3′-diaminobenzidine tetrahydrochloride) biotin avidin detection kit. ISH results were evaluated in a blind manner from coded slides without previous information on clinical history. ISH was considered positive when the hybridization signal was visible as cytoplasmic reactivity in the bronchial epithelial cells. The specificity of the ISH signal was verified by analysis with a probe in sense orientation and by *in vitro* infection of HeLa cells with rhinovirus 14. Uninfected HeLa cells were negative both by the antisense and sense probes (data not shown).

Statistical analyses

For comparing group means, either the Student *t* test or the Mann-Whitney *U* test was used, depending whether the distributions were normal or nonnormal. χ^2 Test was used to compare differences between categorical variables. All reported *P* values are 2-sided, and *P* < .05 was considered statistically significant. Statistical analyses were performed with SPSS 12.0.1 for Windows software (SPSS, Inc, Chicago, Ill).

RESULTS**Infants**

Bronchoscopy was performed in 68 of 201 (34%) infants studied with plethysmography, and biopsies for HRV-ISH were available from 59 of these patients. Twelve biopsies were insufficient; thus, the number of

TABLE I. Characteristics of 47 infants and rhinovirus detection from endobronchial biopsies

	Rhinovirus ⁺ (21)	Rhinovirus ⁻ (26)	P value
Age			
Median, mo	10	10	NS
Range, mo	4 – 23	4 – 26	
Sex			
Male	14 (67%)	17 (65%)	NS
Atopy			
SPT ⁺ and/or atopic eczema	13 (62%)	15 (58%)	NS
SPT ⁺	9 (43%)	5 (19%)	NS
Symptom			
Wheeze ever	18 (86%)	17 (65%)	NS
Wheeze frequently*	11 (52%)	11 (42%)	NS
Wheeze between colds	11 (52%)	10 (38%)	NS
Cough ever	16 (76%)	17 (65%)	NS
Duration of symptoms			
Median, % of age	49%	60%	NS
Range, % of age	26% to 100%	19% to 100%	
Respiratory infection			
6 wk before biopsy	9 (43%)	4 (15%)	.036**
0-6 wk after biopsy	11 (52%)	11 (42%)	NS
Time from lung function test to biopsy			
Median, days	14 (5-65)	21 (7-111)	NS

NS, Nonsignificant.

*Wheeze frequently: 3 or more separate occasions.

**Significant difference between previous respiratory infection and HRV⁺ detection.

specimens for HRV-ISH analysis was 47. The characteristics of these infants are shown in Table I. Bronchoscopy was performed, on average, 20 days (range, 5-111 days) after measurement of lung function.

HRV detection

Human rhinovirus was detected in 21 of 47 infants (45%). In a 3-category setting according to the hybridization signal (strong-moderate-negative), 16 of the positive biopsies (76%) were scored as strong and 5 (24%) as moderate. Fig 1 shows an example of a strong positive and a negative specimen. HRV reactivity was predominantly seen in the respiratory epithelium, but inflammatory cells at the submucosa were also positive.

HRV-ISH⁺ bronchial biopsies were detected throughout the year (Fig 2). The quality or duration of the recurrent respiratory symptoms did not correlate with HRV positivity. In contrast, a significant correlation ($P = .036$) between respiratory infection within 6 weeks before bronchoscopy and positive HRV staining was seen (Table I).

During the follow-up period of 6 weeks after the bronchoscopy, there were 10 clinical exacerbations in both the HRV⁺ and the HRV⁻ groups, whereas nebulized salbutamol or oral corticosteroids were needed during 3 exacerbations in HRV⁺ and during 1 exacerbation in HRV⁻ infants.

Lung function

The lung function, as indicated by sGaw, was normal in 14 of 47 infants (30%). There was a significant correlation between the positive HRV staining and lung function ($P = .037$): 18 of 21 (86%) HRV⁺ infants had abnormal lung

function, compared with 15 of 26 (58%) HRV⁻ infants (Table II). A bronchodilator reversibility was found in 15 infants (32%) but equally in HRV⁺ and HRV⁻ infants. On the other hand, 7 of 9 of those HRV⁺ infants who had a preceding respiratory infection had low sGaw.

Markers for atopy

None of the markers for atopy including atopic eczema, SPT, blood eosinophil count, total IgE, and ECP was associated with HRV reactivity (Tables I and II).

The group of 12 infants with insufficient biopsy material did not differ in any demographic or clinical marker from the study group.

DISCUSSION

We detected HRV by ISH from the lower respiratory tract in 45% of infants with recurrent respiratory symptoms. A positive HRV result correlated with abnormal lung function and occurrence of a respiratory infection during the preceding 6 weeks.

We used HRV-14 probe, which consists 249 nucleotides (nt 332-572) from 5'-untranslated region, and these nucleotide sequences have been shown to be very similar in all HRVs.²⁵ HRV-14 represents genetically the HRV B-species (25 serotypes),²⁶ and it is probable that the probe recognizes at least the members of this cluster, although it was not tested. Because we used only 1 HRV probe, it is possible that combination of HRV probes covering several genotypes could have increased the number of positive samples. Because the biopsies were

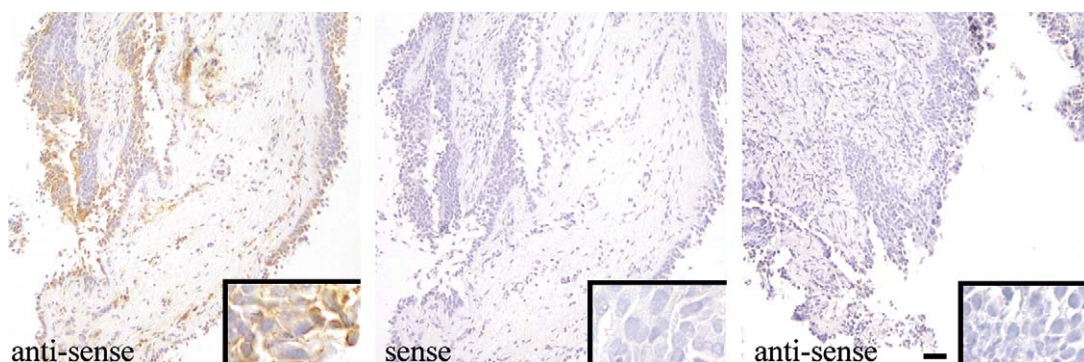


FIG 1. In situ hybridization for HRV-14 in endobronchial tissue. *Left*, HRV-14 detected with antisense probe. Hybridization signal is visible as cytoplasmic brown reactivity in respiratory epithelium and in occasional inflammatory cells. The insert shows epithelial cells at higher magnification. A consecutive section from same specimen with HRV-14 sense probe without any hybridization signal. *Right*, Antisense hybridization of a negative specimen. Bar = 50 μ m.

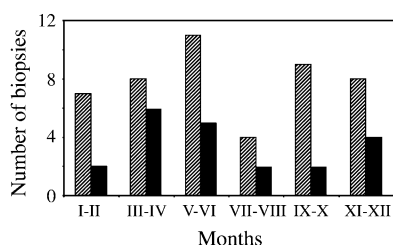


FIG 2. The seasonal distribution of HRV⁺ endobronchial biopsies detected with ISH. *Shaded bars*, all biopsies; *solid bars*, HRV⁺ biopsies.

small, the whole biopsies were placed into formalin and embedded in paraffin, whereafter any PCR analysis was not possible. The lack of culturing for HRV is acknowledged.

Only a few studies have evaluated the presence of viruses in the lower respiratory tract by direct methods and none specifically in small children.^{9,13} The ISH reactivity seen mainly in epithelial and occasionally in inflammatory cells in the bronchial biopsies was in line with studies from other anatomic regions^{24,27} as well as from bronchial biopsy specimens from adults.¹⁰ There is evidence that HRV infects epithelial cells in the upper airways in a patchy rather than diffuse distribution.²⁸ Taking this and the small size of the specimens into account, the negative sample may even represent a clean area between the infected patches rather than true negative sample. In this case, the true number of positive samples could also be even bigger. In a recent study, HRV was detected from a bronchial biopsy by immunohistochemistry in 15 of 19 adults and by PCR in 3 of 19 other subjects after nasopharyngeal experimental infection.¹³ Because ISH used in the current study detects the viral genome within the cells, any contamination of a biopsy from the upper airways can be excluded.

Human rhinovirus causes infections throughout the year, but in the northern hemisphere, peak prevalence occurs in autumn and late spring.^{5,6,29} In the current study, HRV⁺ samples were detected throughout the year.

Respiratory viruses are dominant risk factors for wheezing leading to hospitalization during the first 3 years of life.^{5,6} The frequency of HRV⁺ infants (45%) in the current study is similar to that reported by Rakes et al.³ They detected HRV by PCR from nasal aspirates in 9 of 22 (41%) of wheezing and in 7 of 17 (41%) of nonwheezing control children younger than 2 years at the emergency department. We acknowledge that our patients differed from most infants with relatively mild wheeze and/or cough, who would not normally undergo bronchoscopy. On the other hand, because the infants had been symptomatic for a long period (median, 6 months), the lower airways may have become more vulnerable to infective microbes. The absence of an asymptomatic age-matched control group is an unavoidable weakness of this study. Because of ethical considerations, bronchoscopy cannot be performed solely for research purposes, although the performance of endobronchial biopsy is safe when the procedure is performed by experienced personnel at a tertiary care center.^{21,30,31}

An aberrant immune response to HRV among atopic patients with asthma has been suggested to lead to suboptimal viral clearance and viral persistence.^{19,20} *In vitro* replication of HRV in primary human bronchial epithelial cells has been shown to peak in 24 to 48 hours, but replicating viruses are still present 8 days after inoculation.⁹ The persistence of rhinoviruses and enteroviruses in the upper airways was recently studied by PCR from nasal secretions from children (3 months to 15 years; median, 1.7 years) hospitalized for acute wheeze.³² On admission, 84 samples from 161 children were positive: 26 (16%) for HRV, 29 (18%) for enterovirus, and 29 (18%) for nontypable rhinocytovirus. HRV remained positive in 13 of 26 (50%) cases after 2 weeks and in 1 patient even after 5 weeks. No data on symptoms were collected.

The persistence of HRV in the lower airways after experimentally induced nasal infection was studied from 10 adult volunteers (3 with asthma) by ISH.⁹ Bronchial biopsies were taken before, 3 days after, and 6 to 8 weeks after inoculation. Positive signals for HRV were detected

TABLE II. Lung function, blood eosinophils, total IgE, ECP, and rhinovirus detection in 47 infants with endobronchial biopsies

	Rhinovirus ⁺ (21)	Rhinovirus ⁻ (26)	P value
Normal lung function (n)	3 (14%)	11 (42%)	
Abnormal lung function (n)	18 (86%)	15 (58%)	.037*
Bronchodilator reversibility	8 (38%)	7 (27%)	NS
Sgaw			
Median (kPa ⁻¹ s ⁻¹)	1.6	2.2	NS
Range (kPa ⁻¹ s ⁻¹)	0.7 to 3.6	0.7 to 4.2	
Sgaw			
Median (z scores)	-2.8	-1.8	NS
Range (z scores)	-5.0 to 2.3	-5.5 to 3.8	
FRC			
Median (mL)	277	267	NS
Range (mL)	197 to 394	199 to 390	
FRC			
Median (z scores)	0.22	0.11	NS
Range (z scores)	-1.4 to 3.4	-1.8 to 3.2	
Eos abs			
Median (E9/L)	0.26	0.20	NS
Range (E9/L)	0.07 to 2.92	0.04 to 1.39	
Total			
IgE Median (kU/L)	17	13.5	NS
Range (kU/L)	2 to 218	2 to 669	
ECP			
Median (μg/L)	6.1	6.3	NS
Range (μg/L)	2 to 52.3	2 to 42.9	

Eos abs, Absolute eosinophils; NS, nonsignificant.

*Significant difference between HRV⁺ and HRV⁻ infants in lung function ($P = .037$). χ^2 Test.

in 5 subjects during the infection and in 1 symptomless subject during convalescence. In the current study, 9 of 21 (43%) of HRV⁺ infants had acute respiratory infection during the 6 weeks before the biopsy (on average, 18 days before; range, 5-37 days). It appears that HRV can persist in both the upper and lower airways for several weeks after an acute natural or experimental infection.

There are risk factors for an increased response to HRV infection. The presence of allergy and an increased bronchial responsiveness to histamine as risk factors for enhanced response during experimental HRV infection were studied in subjects with or without allergic (SPT⁺) rhinitis.¹⁶ A low baseline lung function and allergy were suspected to contribute to the increased lower airway effect (increased histamine responsiveness) of HRV infection. In the current study, an abnormal lung function was seen in infants who were HRV⁺. Unfortunately, this cross-sectional study cannot determine which came first, the lung function abnormality or the viral infection. We acknowledge that there was a long delay between the lung function analysis and bronchoscopy (median, 20 days; range, 5-111 days), and the median delay was even 1 week longer in the HRV⁻ group (21 days) than in the HRV⁺ group (14 days). It is possible that the association between HRV detection and abnormal lung function could have been influenced by this delay.

The relationship between atopy and viral respiratory tract infections has been found to be associated with wheezing during childhood. Hospitalization for HRV-

induced wheeze in infancy was shown to be associated with early school-age asthma, and HRV infections were associated with the presence of atopic dermatitis in infancy.⁴ Both in infants⁸ and in older children (>2 years),^{3,33} atopy and HRV infection synergistically promote wheezing. One could argue that HRV is one of the major respiratory pathogens particularly selecting atopic individuals prone to wheezing. In the current study, however, no significant association between atopy, detected by skin prick test, and HRV⁺ was found (Table I). However, the percentage of positive tests in the HRV⁺ and HRV⁻ groups (43% and 19%, respectively) is notable and might well be significant if a larger number of patients were included. The low age of the study infants could partly explain the low number of SPT⁺ patients.

Papadopoulos et al²⁰ studied the balance between type 1 and type 2 immune responses to HRV by incubating PBMCs from atopics and nonatopics with HRV. Incubation of PBMCs with HRV resulted in production of IL-10 in atopic subjects, whereas nonatopic subjects produced IFN- γ and IL-12. Such defective type 1 immune response to HRV in predisposed patients with asthma may contribute to the development of acute asthma exacerbations by promoting type 2 inflammation and diminishing viral clearance through deficient type 1 antiviral immune responses. Very recently, variations in immunologic responses already at birth have been shown to be indicative of the risk for wheezing in high-risk infants during the first year of life.³⁴

To conclude, we have shown in this study that HRV can frequently be found in the lower respiratory tract in these selected infants with recurrent respiratory symptoms. Positive HRV findings correlated with abnormal lung function and previous respiratory infection in the study infants. Clinical follow-up of the patients will reveal whether the HRV positivity during infancy predicts subsequent childhood asthma.

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