The Presence of Rhinovirus in Lower Airways of Patients with Bronchial Asthma

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Rationale: The common cold virus, human rhinovirus (HRV), is the most frequent cause of asthma exacerbations. However, a possible contribution of HRV to the pathogenesis of chronic, persistent asthma has not been defined.

Objectives: To determine if patients with stable asthma, who are free of clinical signs of a respiratory infection for at least 3 weeks, harbor HRV in their bronchi more frequently than nonasthmatic control subjects, and whether clinical features of asthma are associated with the presence of HRV.

Methods: Immunohistochemistry and the indirect *in situ* reverse transcription–polymerase chain reaction method were used to detect the presence of HRV in bronchial mucosal biopsies in patients with asthma and nonasthmatic control subjects.

Measurements and Main Results: HRV was found by immunohistochemistry in 9 of 14 bronchial biopsies from subjects with asthma (64.3%) and 2 of 6 nonasthmatic control subjects (33.3%) ($P = 0.38$). With the more sensitive indirect *in situ* reverse transcription–polymerase chain reaction method, HRV was found in the mucosal biopsies of 73% of patients with asthma and 22% of nonasthmatic control subjects ($P < 0.001$). Subjects positive for HRV had lower pulmonary function, higher numbers of blood eosinophils and leukocytes, and eosinophilic infiltration in bronchial mucosa.

Conclusions: HRV was detected in the lower airway tissue of patients with asthma significantly more often than in nonasthmatic subjects, and its presence was associated with clinical features of more severe disease.

Keywords: asthma; infections; rhinovirus

Respiratory infections with the common cold virus, human rhinovirus (HRV), can have profound effects on asthma (1–4). In an at-risk population of children for asthma, the occurrence of a symptomatic HRV infection during infancy was identified as a significant risk factor for wheezing at the age of 3 years (5). HRV infections are also the major cause of acute exacerbations of wheezing in children and adults with existing asthma (6, 7). Because infections with HRV are transitory for most individuals, their association with persistent asthma has not fully been explored nor has the possibility that a chronic infection of the lower airway with HRV may contribute to the severity of disease. To begin to evaluate the hypothesis that HRV can become

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

There is no previous information to indicate that rhinovirus may be a chronic infection in the lower airways of patients with asthma.

What This Study Adds to the Field

These observations suggest that rhinoviruses can become a chronic infection and contribute to the persistence and severity of airway disease.

a persistent infection of the lower airway and contribute to the severity of asthma in patients with chronic disease, the following study was undertaken. Bronchial mucosal tissue was obtained from patients with chronic stable asthma and nonasthmatic control subjects. These airway mucosal samples were then evaluated for the presence of HRV. Initially, immunohistochemistry (IHC) was used to detect HRV. After these preliminary assessments, a more sensitive technique was developed to detect HRV, *in situ* reverse transcription–polymerase chain reaction (RT-PCR), and this approach was then applied to airway samples from a larger group of patients with asthma and nonasthmatic control subjects. The detection of HRV in airway biopsies was analyzed in relationship to pulmonary functions and measures of inflammation. Our findings from these investigations are reported here.

Part of this study was presented at the Annual Meeting of American Allergy Asthma and Immunology in Miami, Florida (14).

METHODS

Subject Characteristics

Patients were enrolled from February 2003 to October 2005. The recruitment was based on a diagnostic bronchoscopy requested by a specialist not involved in the study. The criteria for diagnostic fiberoptic bronchoscopy were as follows: cough, chest roentgenogram abnormalities ($n = 29$), episodic hemoptysis, or localized wheezing. All subjects who met the study criteria gave their informed, written consent to participate. Asthma was diagnosed according to GINA (Global Initiative for Asthma) clinical criteria (8). All patients with asthma were treated in the University Hospital Department of Medicine, Krakow, Poland, for at least 1 year and had documented FEV₁ values of less than 80% predicted, reversibility after bronchodilators ($>12\%$ change in FEV₁), and/or airway hyperreactivity to methacholine (PC₂₀ < 8 mg/ml). Pulmonary function parameters, atopy, and smoking habits were recorded. Atopy was defined by a history of allergic symptoms and at least one positive skin test to common aeroallergens or highly elevated serum total IgE (>400 IU/ml). A diagnosis of aspirin-induced asthma was based on at least one hospital admission due to life-threatening reaction to nonsteroidal antiinflammatory drugs (NSAIDs) and confirmed, in all except two patients who refused the test, by oral or bronchial challenge (16) with acetylsalicylic acid. The remaining patients with asthma gave a history of tolerance of NSAIDs; aspirin provocation

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tests were performed in half of these subjects and all were negative. Control subjects were recruited during fiberoptic bronchoscopy performed to evaluate hemoptysis, peripheral lung tumor, or dyspnea (although in these last two situations, diagnostic procedures had shown no abnormalities); or, control subjects were nonasthmatic individuals who were enrolled from the District Hospital Department of Thoracic Surgery, Krakow, Poland, where they had been admitted for a scheduled lobectomy or pneumonectomy due to a primary peripheral lung malignancy. Lung tumor was present in two of six control subjects evaluated by IHC and in 16 of 23 control subjects in whom *in situ* RT-PCR was used. All surgical specimens were verified by a pathologist and chronic obstructive pulmonary disease (COPD) was ruled out.

Exclusion criteria for the subjects with asthma were a recent exacerbation, defined as an increase in subjective symptoms and/or worsening of pulmonary function tests during the 2-week period preceding the screening visit, or symptoms of an upper respiratory illness during the 3 weeks preceding the screening visit. Control subjects with symptoms of common cold during the 3 weeks preceding the screening visit or a chronic lung disease (i.e., COPD, bronchiectases, or emphysema) were excluded from study. Subjects with primary lung malignancies were excluded when the disease was disseminated or locally advanced, such that there was a possibility of neoplastic infiltrations of bronchial biopsies.

Mucosal biopsies were obtained by fiberoptic bronchoscopy in all subjects with asthma. Eleven control subjects also had samples obtained during bronchoscopy. Fifteen nonasthmatic control subjects underwent pulmonary surgery, and their mucosal samples were analyzed by *in situ* RT-PCR only. Mucosal biopsies from 20 individuals, including 14 subjects with asthma and 6 control subjects, were evaluated by the IHC method, and 53 subjects, including 30 subject with asthma and 23 control subjects, were evaluated by *in situ* RT-PCR. Twelve subjects of this group, nine with asthma and three control subjects, were evaluated by both methods. Three aspirin-sensitive subjects with asthma were evaluated by IHC, and seven aspirin-sensitive patients with asthma were evaluated by the *in situ* indirect RT-PCR procedure.

The study was approved by the Jagiellonian University School of Medicine Human Subjects Ethics Committee, and informed consent was obtained from all participants after an explanation of additional procedures planned for research purposes.

Bronchial Biopsies

The procedure was performed at the Jagiellonian University School of Medicine, according to the guidelines of the British Thoracic Society (9). Bronchoscopies were performed transnasally by the same operator. During the procedures, three to five sections of bronchial mucosa were taken using microforceps from a macroscopically normal segmental bronchus or carina minor. Usually, samples were obtained from the carina minor on the right side or middle-lobe bronchus. The area was selected for a normal appearance by color, surface, and vasculature. Bronchial biopsies were immediately fixed in 4% formaldehyde and refrigerated until processed. During the lobectomy or pneumonectomy, fragments of segmental bronchi were taken from the excised lung. The surgeon sampled a segmental bronchus that was macroscopically normal and located distally to the tumor. The excised tissue sample was immediately fixed in 4% formaldehyde and refrigerated before paraffin embedding and microtome sectioning by the same process used with biopsies sampled during bronchoscopy.

Pulmonary Function Tests

Parameters of FVC, FEF₅₀ (forced expiratory flow at 50% of vital capacity), and FEV₁ were measured using the PNEUMO 2000 (abcMED, Krakow, Poland) computerized spirometer by the same experienced operator.

IgE Levels

Total serum IgE level was measured by laser immunonephelometry (Dade Behring Nephelometer Analyser II; Marburg, Germany).

Peripheral Blood Cell and Eosinophil Counts

Peripheral blood cells were counted using a Sysmex K-1000 apparatus (Sysmex, Kobe, Japan). Eosinophils were counted manually using a light microscope, after dilution of the whole blood in eosin solution.

Tissue Preparation and IHC Staining

The biopsies sampled during bronchoscopy or taken from excised lung tissue were paraffin embedded and sectioned. HRV-infected cells were identified using monoclonal antibody RV16-7 as described elsewhere (10). This antibody was shown to bind the capsid protein VP2 of HRV16 and HRV1A, but not HRV14 or HRV2; however, no additional serotypes were tested. Eosinophils in bronchial mucosa sections were stained with monoclonal antibody EG2 (Pharmacia, Uppsala, Sweden). Times of incubation and dilutions of antibodies EG2 and RV16-7 were adjusted experimentally. Positive controls included paraffin sections of HRV-infected HeLa cell culture and tonsillar tissue incubated *ex vivo* with HRV. The negative control was a HeLa cell line. The assessment of HRV presence in sections was performed twice by two blinded observers.

Rhinovirus detection and eosinophilic inflammation were scored using at least 10 ×200 magnification fields, using a 0- to 5-point scale, where 0 meant no signal. A positive signal of a single cell expressing HRV, in at least one magnification field, was scored as 1, single cells with HRV in more than three magnification fields were scored as 2, groups of cells in at least one magnification field were scored as 3, and a strong positive signal over more than three magnification fields was scored as 5. The scoring was performed by two unblinded observers. Score grades differed between duplicates in 3 samples and was uncertain in 7 samples out of 20 samples. This was resolved with additional replicates.

Hybridization Probe Synthesis

A nonasthmatic individual with symptoms of an acute common cold served as a source of HRV material. Each nostril was washed and total RNA was isolated. Reverse transcription was performed using AMV (avian myeloblastosis virus) reverse transcriptase (Amresco, Solon, OH) and universal reverse primer, selected from the VirOligo database (OL86-1; Oklahoma State University, Stillwater, OK; <http://virologo.okstate.edu>) and modified to strengthen primer homology to HRV2 and HRV16 sequence. The product of reverse transcription reaction was amplified using PCR methods by the addition of the forward primer (OL24). cDNA was cloned into TOPO PCR II plasmid (Invitrogen, Carlsbad, CA) vector using the TOPO TA cloning kit (Invitrogen). After identification of the insert by a direct sequencing (BigDye version 3.1 kit; Applied Biosystems, Foster City, CA), the probe was generated from the plasmid template by direct labeling with digoxigenin (Dig Labelling Mix; Boehringer Ingelheim, Ingelheim, Germany) during PCR, with the same primers as for cloning.

The specificity of digoxigenine-labeled probe was evaluated by a comparison with a database of viral sequences. The hybridization probe of 536-nucleotide length was aligned by BLAST software to a viral nonredundant database (National Center for Biotechnology Information, Bethesda, MD; www.ncbi.nlm.nih.gov/BLAST) and demonstrated at least 79% identity to 55 different serotypes, eight strains and two isolates of rhinoviruses, recorded in the database. Identified by homology, rhinoviruses belonged to phylogenetically defined type A and unclassified rhinovirus species, but not to HRV type B (20). The probe sequence corresponded to the viral sequence region VP4/VP2 encoding two capsid proteins. Sequence analysis showed the highest 89% nucleotide identity to the human rhinovirus clone HRV24. No homologies between the probe sequence and non-rhinovirus organisms or human genomic sequence were found.

Indirect RT-PCR *In Situ* Hybridization

Paraffin sections were mounted on *in situ* PCR glass slides (Perkin Elmer, Fremont, CA). Reverse transcription was performed using the reverse primer and AMV reverse transcriptase (Amresco) according to the manufacturer's protocol for the thermal cycler GeneAmp *In Situ* PCR System 1000 (Perkin Elmer). Thereafter, the reverse transcription mixture was replaced with a PCR mixture containing Taq polymerase (Finnzymes, Espoo, Finland) and the same primers as were used for the probe. The digoxigenin-labeled and heat-denatured probe (250 ng/ml) was hybridized overnight. The hybridized probe was detected by IHC using anti-DIG Fab fragments conjugated with alkaline phosphatase (Boehringer Ingelheim). Because 17% of sections gave results, which differed in duplicates' scores, additional replicates were processed using indirect *in situ* RT-PCR to resolve any discrepancies.

Positive and negative controls were included in each batch of reactions. The positive control was the section giving positive results in previous experiments. The negative controls, separate for each subject, were sections treated according to the same procedure, except for omission of the reverse primer during the reverse transcription of HRV. The procedure of indirect *in situ* RT-PCR was performed twice for all patients. Each section was assessed twice by a blinded observer. The scoring system of HRV-positive sections was the same as that described for IHC.

Additional data on the molecular procedures used and sequence of the primers and probe are available in the online supplement.

Statistical Methods

Data were analyzed using a personal computer Statistica software (version 5.5; StatSoft, Inc., Tulsa, OK). Unpaired Student *t* test, Pearson's correlation, Mann-Whitney *U* test, or Kendall's rank sum test for parametric and nonparametric data were performed as appropriate. Statistical significance was accepted as *P* less than 0.05.

RESULTS

HRV Detection by IHC

Twenty bronchial mucosal biopsies were stained with RV16-7 antibody. These samples had been obtained from 14 patients with asthma (including 3 subjects with aspirin-sensitive asthma) and 6 control subjects, whose characteristics are described in Table 1. The viral capsid protein was found in 9 of 14 biopsies from patients with asthma and 2 of 6 control subjects. Although there was an increased proportion of HRV-positive staining in asthma as compared with controls, differences were not significant (χ^2 $P = 0.38$). Only one out of three subjects with aspirin-sensitive asthma was HRV positive by IHC. There was, however, a significant correlation between the score of the viral capsid protein staining and eosinophilic infiltration in bronchial samples, scored as an average number of eosinophils per magnification fields ($\times 200$) in mucosal biopsy (Kendall's tau = 0.34, $P < 0.05$). To extend these studies and enhance the likelihood to detect HRV, a more sensitive method was developed, *in situ* RT-PCR.

HRV Detection by *In Situ* RT-PCR

Samples of bronchial mucosa from 30 subjects with asthma, including 7 patients with aspirin hypersensitivity, and 23 control

subjects were evaluated using indirect *in situ* RT-PCR (Table 1). The mean age of subjects with asthma and nonasthmatic subjects differed significantly, but male-to-female ratio and FEV₁ values of subjects with asthma and control subjects were similar. The study groups evaluated by either IHC or *in situ* hybridization did not differ significantly in their clinical features (Table 1).

The indirect *in situ* RT-PCR method gave a strong positive staining in some biopsies compared with negative controls. HRV-positive cells were detected in 73% of biopsies from patients with asthma but only in 22% of biopsies from nonasthmatic control subjects ($P < 0.001$). All biopsies from aspirin-hypersensitive patients with asthma had HRV-positive staining ($P < 0.001$ vs. controls; $P = 0.08$ vs. aspirin-tolerant subjects with asthma). The frequency of positive skin tests to common aeroallergens, total serum IgE, and previous diagnosis of atopic diseases did not differ between HRV-positive and HRV-negative patients with asthma.

The number of subjects in whom both IHC and *in situ* RT-PCR was conducted was limited. Twelve individuals were tested by both methods (9 subjects with asthma and 3 controls) (Table 2). The concordance between IHC and *in situ* RT-PCR was 50%. *In situ* RT-PCR detected viral genome in an additional four subjects and failed to detect HRV in two others, who were positive with IHC.

Patterns of *In Situ* RT-PCR Staining

Two different patterns of staining within bronchial mucosa sections were found with *in situ* RT-PCR. One pattern was patchy and limited to mucosal surface areas with well-delineated borders; this is referred to as localized. The second pattern of staining was diffuse, with a positive signal seen throughout the section without any confined areas. Examples of localized and diffuse staining are shown in Figure 1.

Protease digestion and thermal cycling obscured the morphology in some of these sections, limiting a morphologic analysis of the stained biopsies. In many cases, however, it was still possible to distinguish bronchial epithelium and other mucosal structures. In these samples, the HRV-positive cells were localized both to epithelial and subepithelial layers of mucosa. Interestingly, non-morphologically identified cells, similar to lymphocytes, gave a strong stain signal in some HRV-positive biopsies. In addition, some positive stain structures were morphologically similar to submucosal glands; this was seen in nine patients with representative HRV staining results of the RT-PCR *in situ* method shown in Figure 2.

Clinical Correlations with HRV Detection

Pulmonary functions. A relationship was found between HRV detected by indirect *in situ* RT-PCR and measures of pulmonary function. In both control subjects and subjects with asthma who stained positively for HRV, significantly lower FEV₁ ($P < 0.05$), FEV₁/FVC ($P < 0.01$), and FEF₅₀ values ($P < 0.05$) were found (Table 3). The difference in FEV₁ values between HRV-positive and HRV-negative subjects was also significant when only patients with asthma were compared ($P < 0.05$). When we evaluated these subjects in relationship to aspirin sensitivity, all seven aspirin-sensitive individuals tested positive for HRV (Table 4). Although lung function values tended to be lower in the aspirin-sensitive group, the differences between the aspirin-tolerant and aspirin-sensitive group did not achieve significance, probably reflecting the small number of subjects evaluated.

Peripheral Blood Cell Values

Both subjects with asthma and control subjects with HRV detected had significantly higher peripheral white blood cell and polymorphonuclear leukocyte counts (Mann-Whitney *U* test,

TABLE 1. CLINICAL CHARACTERISTICS OF THE SUBJECTS STUDIED

	Indirect <i>In Situ</i> RT-PCR		Immunohistochemistry	
	Asthma (n = 30)	Controls (n = 23)	Asthma (n = 14)	Controls (n = 6)
Age, yr*	41 ± 13 [†]	54 ± 15	41 ± 10	49 ± 24
Sex, F/M ratio	22/8	14/9	7/7	3/3
Duration of asthma, yr*	6 ± 7	0	10 ± 12	0
Atopy, yes/no (n = 28)	14/14	NA	5/9	NA
Smoking status, yes/no	4/26	7/14	1/13	1/5
Blood eosinophil count, cells/mm ³ *	312 ± 339	NA	400 ± 328	NA
Serum IgE, UI/ml*	354 ± 714	NA	463 ± 819	NA
FEV ₁ , % of predicted value*	83 ± 25	95 ± 18	86 ± 23	99 ± 1
Inhaled corticosteroids, yes/no	27/3 [‡]	1/22	14/0 [‡]	0/6
Patients receiving oral corticosteroids	9/30	2 [‡] /23	2/14	0/6
HRV detected	22/30 [†]	5/23	9/14	2/6

Definition of abbreviations: F/M = female/male; HRV = human rhinovirus; NA = not assessed; RT-PCR = reverse transcription-polymerase chain reaction.

* Values are expressed as means ± SD.

[†] Significant difference ($P < 0.01$) between asthma and control group.

[‡] Low-dose treatment because of arthritis.

TABLE 2. COMPARISON OF PATIENTS STUDIED BOTH BY IMMUNOHISTOCHEMISTRY AND *IN SITU* REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

No./Sex/Age (in yr)	Study Group	Indications for Bronchoscopy	IHC HRV Detection/Score	<i>In Situ</i> RT-PCR HRV Detection/Score	<i>In Situ</i> RT-PCR Staining Pattern	Bronchial Mucosa Eosinophils	Histopathology
1/F/27	Control	Recurrent infections, chest X-ray	Negative/0	Negative/0	NA	0	Chronic bronchitis
2/M/47	Control	Hemoptysis	Positive/3	Negative/0	NA	0	Normal
3/F/55	Control	Hemoptysis	Negative/0	Positive/1	Focal	0	Normal
4/F/35	Asthma	Cough	Positive/3	Positive/3	Diffuse	3	Chronic bronchitis
5/F/51	Asthma	Cough, chest X-ray	Positive/1	Negative/0	NA	0	Chronic bronchitis
6/F/42	Asthma	Cough, chest X-ray	Positive/1	Positive/2	Diffused	1	Chronic bronchitis
7/M/42	Asthma	Cough, chest X-ray	Positive/1	Positive/1	Focal	1	NA
8/M/49	Asthma	Hemoptysis	Positive/1	Positive/1	Focal	0	Chronic bronchitis
9/M/39	Asthma	Cough	Positive/3	Positive/3	Diffuse	4	Chronic bronchitis
10/F/35	Asthma	Cough	Positive/1	Negative/0	NA	0	Normal
11/F/48	Asthma, aspirin-induced	Cough	Negative/0	Positive/1	Focal	2	Chronic bronchitis
12/M/27	Asthma, aspirin-induced	Cough	Negative/0	Positive/3	Diffuse	0	Chronic bronchitis

Definition of abbreviations: F = female; HRV = human rhinovirus; IHC = immunohistochemistry; M = male; NA = not available; RT-PCR = reverse transcription-polymerase chain reaction.

For scores of staining, see METHODS.

$P < 0.05$ and $P < 0.01$) but lower lymphocyte values ($P = 0.001$). These differences between the HRV-positive and HRV-negative groups were also significant when patients with asthma were analyzed separately (Table 3). The aspirin-tolerant asthmatic group demonstrated similar trends to the entire asthma group (Table 4), but the statistical power was not sufficient to fully assess differences due to the limited number of subjects evaluated. For this reason, the only significant difference between the RT-PCR HRV-positive and HRV-negative aspirin-tolerant subjects with asthma was found to be a higher percentage of blood lymphocytes ($P = 0.02$), whereas blood neutrophils and eosinophils showed only trends toward being lower ($P = 0.06$ and $P = 0.07$, respectively; two-tailed significance).

When HRV-positive subjects with asthma and control subjects were analyzed together, the indirect *in situ* RT-PCR signal score also correlated with peripheral blood leukocytes ($P < 0.01$, Kendall's tau = 0.3) and with peripheral blood polymorphonuclear cells ($P < 0.01$, Kendall's tau = 0.36). In addition, there was a positive correlation between the HRV signal score and the percentage of peripheral blood polymorphonuclear cells ($P < 0.001$, Kendall's tau = 0.42). A negative correlation was found between HRV signal score and percentage of peripheral blood lymphocytes ($P < 0.001$, Kendall's tau = -0.41). These correlations between the HRV signal score and peripheral blood cell counts were still significant when patients with asthmas were analyzed separately (Table 5). A significant correlation between score of HRV signal extended to FEV₁ ($P < 0.01$, Kendall's tau = -0.21) and FEV₁/FVC ($P < 0.05$, Kendall's tau = -0.21), but only for subjects with asthma and control subjects analyzed together (Table 5). In the aspirin-tolerant group, the RT-PCR HRV signal score correlated with blood leukocytes (tau = 0.428, $P = 0.04$), blood lymphocyte percentage (tau = 0.650, $P = 0.008$), and blood eosinophils (tau = 0.422, $P = 0.016$).

Peripheral blood eosinophils were increased in subjects with asthma and control HRV-positive subjects (363 ± 364 cells/mm³) compared with HRV-negative subjects (140 ± 132 cells/mm³; Mann-Whitney *U* test, $P < 0.05$). Peripheral blood eosinophils also correlated positively with the HRV signal score (Kendall's tau = 0.28, $P < 0.01$). This correlation was still significant when only patients with asthma were considered (Kendall's tau = 0.42, $P < 0.01$; Table 5).

Mucosal Biopsy Analysis

The HRV indirect *in situ* RT-PCR signal score was also compared with the mucosal biopsy eosinophil values in 14 subjects (11

subjects with asthma and 3 nonasthmatic controls); a significant positive correlation was found (Kendall's tau = 0.51, $P = 0.01$).

Finally, a comparison of all HRV-positive subjects, according to the pattern of staining, revealed a significantly increased peripheral blood eosinophil count in those subjects with a diffuse signal (629 ± 334 cells/mm³) in contrast to the mucosal biopsies with a localized pattern (182 ± 313 cells/mm³; Mann-Whitney *U* test, $P < 0.01$).

DISCUSSION

HRV antigen was found in airway tissue of subjects with asthma and normal subjects in the absence of symptoms of a clinical respiratory infection. HRV, however, was detected more frequently in bronchial mucosal biopsy tissue from patients with asthma than in similar tissue samples from normal control subjects. Although the differences in the presence of HRV in bronchial mucosal samples were predominantly based on RNA detection by *in situ* hybridization, similar trends were seen with antigen recognition by IHC. Moreover, the presence of HRV in lower airway samples had significant associations with clinical characteristics of the subjects evaluated. For example, the presence of HRV correlated with increased levels of circulating polymorphonuclear leukocytes and, in asthma, peripheral blood eosinophils as well as eosinophilic infiltration of the mucosal tissue. In addition, the presence of HRV was associated with a greater degree of airflow obstruction in all subjects. In patients with asthma, lower FEV₁ values were found in those with HRV detected, a possible indicator of greater asthma severity. HRV-positive aspirin-tolerant and aspirin-sensitive patients with asthma did not differ. However, HRV was detected in all of a limited number of aspirin-sensitive patients with asthma tested ($n = 7$). This phenotype of asthma usually has more severe disease, and interestingly, upper respiratory tract infections commonly precede the first symptoms of aspirin-provoked asthma (11, 12). Thus, persistent viral infection remains an attractive hypothesis for the pathogenesis of aspirin-sensitive asthma (13). Finally, in preliminary analysis, we were unable to determine the influence of the use of inhaled corticosteroids on the presence of HRV. Binary logistic regression using presence of HRV ($R^2 = 0.012$) and nonparametric regression using HRV staining score (Kendall's tau = -0.165) on inhaled corticosteroid dose were not significant. In future work, treatment with corticosteroids will be assessed as a potential risk factor for the presence of viruses. Collectively, our results suggest

Figure 1. Examples of a localized (A) and diffuse (B) pattern of staining using *in situ* indirect reverse transcription-polymerase chain reaction detection of human rhinovirus. (A) A 47-year-old woman with lung cancer, rheumatoid arthritis, and arterial hypertension, and who is a smoker; FEV₁ = 80% predicted. (B) A 42-year-old woman with mild-to-moderate asthma, chronic sinusitis, nasal polyposis, and atopy; FEV₁ = 93% predicted, peripheral blood eosinophils = 310 cells/mm³.

that the presence of HRV in lower airway tissue may contribute to asthma severity in some patients.

Our study design does not allow us to determine whether the presence of HRV reflects a persistent infection or a single isolated event. Although we were careful to exclude patients who had had symptoms of a respiratory infection in the past 3 weeks, this time frame may not totally exclude the possibility of a recent HRV illness (14). With experimental HRV infections, virus can persist in the lower airways for more than 3 weeks in some subjects, even though the patients are free of cold symptoms (15–18). In our study, however, HRV detection was greater in subjects with asthma than in normal control subjects, suggesting a feature more likely linked to asthma to explain the presence of virus and a respiratory disease in which defects in the immune response to HRV may compromise virus clearance (19, 20). Furthermore, the possibility that rhinoviruses can cause long-term, persistent infections has recently been reported by Kaiser and coworkers

(21). In pulmonary tissue samples from lung transplantation patients, these investigators have shown that HRV can persist in the airway for long periods of time and contribute significantly to the respiratory disease. Therefore, further longitudinal, long-term studies in asthma are needed to determine whether HRV persists in the lung and if its presence is a factor in disease severity.

We did not test for the presence of other respiratory pathogens that have been linked to the persistence and severity of asthma (22, 23). Our focus on rhinovirus infections, however, was based on the significant role these microorganisms play in many aspects of asthma (1–4). In infancy, rhinovirus infections in the first year of life are important risk factors for recurrent wheezing at 3 years of age (5). Moreover, in both children and adults, rhinovirus infections are the most frequent cause of asthma exacerbations, and may lead to increased asthma severity after the acute respiratory illness (6, 7). Furthermore, there is emerging evidence

Figure 2. Representative staining of bronchial biopsies with indirect *in situ* reverse transcription-polymerase chain reaction. (A) Human rhinovirus (HRV) probe hybridization signal in the epithelial layer of bronchial mucosa in a 52-year-old nonsmoking woman with chronic cough and FEV₁ of 96% predicted; initial suspicion of asthma was excluded. (B) HRV hybridization probe signal in the epithelial layer of bronchial mucosa of a 62-year-old woman smoker with aspirin-sensitive severe asthma; FEV₁ = 34% predicted. During the study, she received oral methylprednisolone. (C) Unidentified subepithelial cells detected with the HRV hybridization probe in a 22-year-old woman nonsmoker with mild-to-moderate asthma and allergic rhinitis; FEV₁ = 106% of predicted and blood eosinophils = 160 cells/mm³. (D) HRV hybridization probe detected rounded submucosal structures, morphologically similar to subepithelial glands in a 27-year-old woman with nonallergic, mild-to-moderate asthma, chronic sinusitis, nasal polyposis, and rhinopharyngitis; FEV₁ = 112% of predicted and blood eosinophils = 59 cells/mm³. (E) Another example of submucosal structures morphologically similar to subepithelial glands in a 59-year-old woman with aspirin hypersensitivity, mild-to-moderate asthma, chronic sinusitis, and who is a nonsmoker; FEV₁ = 103% of predicted value and blood eosinophils = 396 cells/mm³. (F) HRV-negative rounded submucosal structures, morphologically similar to subepithelial glands, in the same patient as shown in (E); figure shows specificity of HRV signal in submucosal structures.

TABLE 3. RELATIONSHIPS BETWEEN DETECTION OF HUMAN RHINOVIRUS AND PULMONARY FUNCTION OR PERIPHERAL BLOOD CELL COUNTS: *IN SITU* REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION DETECTION ONLY

Parameter	Asthma		Controls		Asthma and Controls	
	HRV+	HRV–	HRV+	HRV–	HRV+	HRV–
FEV ₁ % (SD)	78.6 (26.2)	94.1 (18.1)	84.1 (20.3)	99.3 (15.5)	79.5 (25.0)*	97.0 (16.5)
FVC% (SD)	91.1 (20.1)	96.9 (18.2)	86.7 (21.3)	99.4 (20.0)	90.4 (19.9)	98.4 (18.8)
FEV ₁ /FVC (SD)	71.0 (14.8)*	81.1 (8.3)	79.8 (1.5)	84.0 (8.3)	72.5 (13.9)*	82.8 (8.2)
FEF _{25%} (SD)	71.0 (30.6)	89.4 (31.3)	NA	104.9 (13.2)	72.8 (29.6)	94.6 (26.8)
FEF _{50%} (SD)	57.5 (23.5)	74.8 (23.2)	NA	85.3 (7.29)	57.5 (22.7)*	78.3 (19.4)
FEF _{75%} (SD)	53.6 (28.5)	63.8 (22.9)	NA	77.5 (14.5)	52.6 (27.7)	68.4 (20.7)
Blood leukocytes, ×10 ³ /mm ³ (SD)	8.4 (2.2)	6.8 (2.3)	7.6 (4.2)	6.7 (1.6)	8.2 (2.6)*	6.8 (1.8)
Blood lymphocytes, ×10 ³ /mm ³ (SD)	1.9 (0.6)	2.1 (0.6)	1.8 (0.6)	2.4 (1.1)	1.9 (0.6)	2.3 (1.0)
PML, ×10 ³ /mm ³ (SD)	5.9 (2.2)*	4.3 (1.9)	5.4 (3.7)	3.7 (1.3)	5.8 (2.5)*	3.9 (1.5)
Blood lymphocytes, % (SD)	23.3 (7.7)*	31.0 (4.2)	26.5 (10.6)	35.9 (13.9)	24.0 (8.2)*	34.2 (11.5)
PML, % (SD)	69.3 (8.9)*	61.9 (5.7)	66.2 (13.4)	54.4 (13.1)	68.5 (9.8)*	57.0 (11.5)
Blood eosinophils, ×10 ³ /mm ³ (SD)	393.2 (370.7)*	119.4 (116.7)	80.0 (29.7)	156.5 (147.4)	363.4 (364.1)*	140.0 (132.1)

Definition of abbreviations: HRV = human rhinovirus; NA = not assessed; PML = polymorphonuclear leukocytes.

Differences between HRV-positive vs. -negative subjects were tested within subjects with asthma and all subjects using Mann-Whitney *U* test.

* *P* < 0.05.

that abnormalities in interferon generation to rhinovirus exist in asthma; these antiviral deficiencies may be an important risk factor for the susceptibility of some patients with to respiratory viruses; and the altered interferon generation may serve as a possible determinant of airway obstruction during the respiratory illness (14, 19, 20). These factors and associations with asthma prompted us to target rhinovirus in our initial investigations.

Compared with other studies in asthma (24–26), our ability to detect HRV in lower airway tissue was greater and may relate to the use of the indirect RT-PCR *in situ* hybridization method. The sensitivity of the indirect *in situ* RT-PCR is at least as great as standard PCR methods and an estimated eight times more sensitive than hybridization alone (27), including analysis of biopsy specimens (28). Moreover, *in situ* RT-PCR has the advantage over the classical RT-PCR method, as the viral

genome is detected within the infected cells. This feature can minimize a false-positive signal due to a contamination of the biopsy sample with material originating from the upper respiratory airways. All patients in our study were sampled using transnasal bronchoscopy, whereas two-thirds of the control subjects were evaluated using *in situ* RT-PCR with samples taken by surgery. We believe that hybridization, which localized HRV RNA within the cellular structures of the biopsy, made the possibility of an artifactual transfer of the viral material from the upper airway during bronchoscopy unlikely. In addition, the wide spectrum of RT-PCR amplification targets was limited by the HRV-specific hybridization probe, which was complementary to HRV but not other picornaviruses, and still enabled detection of a substantial range of HRV serotypes. Moreover, the probe had enough complementarity during the hybridization process to detect specifically only HRV, but this did not preclude detection

TABLE 4. RELATIONSHIP BETWEEN DETECTION OF HUMAN RHINOVIRUS AND PULMONARY FUNCTION OR PERIPHERAL BLOOD CELL COUNTS IN ASPIRIN-TOLERANT AND ASPIRIN-SENSITIVE SUBJECTS WITH ASTHMA

Parameter	Aspirin-tolerant Asthma (<i>n</i> = 23)		Aspirin-sensitive Asthma (<i>n</i> = 7)
	HRV+ (<i>n</i> = 15)	HRV– (<i>n</i> = 8)	HRV+ (<i>n</i> = 7)
FEV ₁ % (SD)	83.1 (23.2)	94.1 (18.1)	68.3 (31.9)
FVC% (SD)	92.6 (18.2)	96.9 (18.2)	87.6 (25.5)
FEV ₁ /FVC (SD)	74.9 (11.7)	81.1 (8.3)	61.8 (18.3)
FEF _{25%} (SD)	81.7 (30.3)	89.4 (31.4)	54.2 (24.2)
FEF _{50%} (SD)	63.6 (23.4)	74.8 (23.3)	46.5 (21.6)
FEF _{75%} (SD)	60.1 (33.1)	63.8 (22.9)	41.8 (13.8)
Blood leukocytes, ×10 ³ /mm ³ (SD)	7.9 (2.2)	6.8 (2.3)	9.4 (2.2)
Blood lymphocytes, ×10 ³ /mm ³ (SD)	1.9 (0.7)	2.1 (0.6)	1.8 (0.6)
PML, ×10 ³ /mm ³ (SD)	5.4 (1.6)	4.3 (1.5)	7.0 (3.2)
Blood lymphocytes, % (SD)	24.3 (6.0)	31.0* (4.2)	21.5 (10.8)
PML, % (SD)	67.7 (5.4)	61.9 (5.7)	72.7 (14.8)
Blood eosinophils, ×10 ³ /mm ³ (SD)	343.8 (388.8)	119.0 (116.7)	500.2 (334.2)

Definition of abbreviations: HRV = human rhinovirus; PML = polymorphonuclear leukocytes.

* *P* = 0.02 for comparison between reverse transcription-polymerase chain reaction (RT-PCR)-positive and RT-PCR-negative aspirin-tolerant patients.

TABLE 5. NONPARAMETRIC CORRELATIONS OF *IN SITU* INDIRECT REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION SIGNAL SCORE AND LUNG FUNCTION OR PERIPHERAL BLOOD LEUKOCYTOSIS

Parameter	Asthma Median, 1.5 (0–4)		Controls Median, 0 (0–5)		Asthma and Controls Median, 1 (0–5)	
	tau	<i>P</i> Value	tau	<i>P</i> Value	tau	<i>P</i> Value
FEV ₁ %	–0.04	NS	–0.34	0.08	–0.21*	0.005
FVC%	0.006	NS	–0.20	NS	–0.11	NS
FEV ₁ /FVC	–0.10	NS	–0.30	NS	–0.21*	0.038
FEF _{25%}	–0.02	NS	–0.71	NS	–0.13	NS
FEF _{50%}	–0.08	NS	–0.71	NS	–0.18	NS
FEF _{75%}	–0.11	NS	–0.71	NS	–0.22	NS
Blood leukocytes, ×10 ³ /mm ³	0.41*	0.009	0.02	NS	0.30*	0.008
Blood lymphocytes, ×10 ³ /mm ³	–0.006	NS	–0.37*	0.04	–0.19	0.10
PML, ×10 ³ /mm ³	0.48*	0.004	0.09	NS	0.36*	0.002
Blood lymphocytes, %	–0.44*	0.007	–0.27	0.12	–0.41*	0.0004
PML, %	0.36*	0.03	0.32*	0.07	0.42*	0.0003
Blood eosinophils, ×10 ³ /mm ³	0.42*	0.003	–0.13	NS	0.28*	0.005

Definition of abbreviations: NS = not significant; PML = polymorphonuclear leukocytes.

In situ indirect reverse transcription-polymerase chain reaction signal score is reported within each group as median (range)

* *P* < 0.05.

of unknown viruses sharing a similar VP2 and VP4 coding region. Thus, it is possible that other serotypes, or serotypes yet to be fully identified, were not detected in these samples, and the true incidence of HRV in samples might be underestimated (29). Finally, our methods did not quantitate the amount of HRV in the sample. In other studies in asthma, the viral load in the airway is associated with acute symptoms and severity, as well as the likely effect on lower airway dysfunction (20). To fully ascertain the significance of rhinovirus to persistent asthma, all of these variables need to be addressed.

The detection of HRV correlated with clinical features in patients with asthma recruited for study. Significantly lower pulmonary functions were found in the HRV-positive patients. This association parallels findings of Malmström and colleagues, who also found lower lung function in children who had HRV persist in airway samples (30). Lower rates of airflow were also found in children with asthma who harbored HRV 6 weeks after an acute illness than in those who had cleared this virus (16). The significance of HRV to more severe asthma was not established in our study, including in those subjects with aspirin sensitivity, but may relate to the contribution of HRV to bronchial inflammation (2).

The detection of HRV also correlated with circulating leukocyte patterns associated with acute colds. Higher peripheral blood polymorphonuclear cells were found in patients with HRV detected. Acute HRV infections cause an increase in circulating blood, nasal, sputum, and bronchoalveolar lavage neutrophils, which is likely the result of this respiratory virus stimulation of neutrophil chemoattractants such as IL-8, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor (14, 15, 17, 31). HRV-positive subjects also had an increase in peripheral blood eosinophils and eosinophilic infiltration of mucosal biopsies. Fraenkel and coworkers (32) also found an increased and persistent eosinophilic infiltration of the bronchial mucosa after the common cold. Under some conditions, HRV infection can stimulate local cytokine production of eotaxin, eotaxin-2, RANTES (regulated upon activation, normal T-cell expressed and secreted), and granulocyte-macrophage colony-stimulating factor to attract and stimulate eosinophils (15). Moreover, we did distinguish different patterns of HRV probe staining, diffuse versus localized, in relationship to tissue eosinophils. Patients with a diffuse pattern had a higher number of eosinophils than seen with a localized pattern. Whether this relationship represents a greater viral load and generation of chemoattractant factors was not ascertained. Overall, the similarities of these changes in peripheral blood values to events in acute rhinovirus infections support a clinical relevance of HRV detection in airway tissue samples to a possible ongoing infection.

Finally, the HRV probe localized to not only bronchial epithelial cells but also to unidentified solitary cells in submucosal tissue. A similar observation was reported by Papadopoulos and colleagues (15), who described positive staining of unidentified subepithelial bronchial cells after hybridization *in situ*. In several biopsies, rounded subepithelial structures resembling glandular acini were stained by the HRV probe, supporting the possibility of a persistence of infection in the lumen of glands, where mucociliary clearance of virus is not sufficient. The pattern of such staining was repetitive and was found not only in subjects with asthma but also in control subjects. Some previous reports documented that HRV could infect submucosal glands and lead to production of cytokines and eosinophil chemotactic mediators (33, 34).

In summary, our results suggest that a substantial number of patients with stable asthma, and free of clinical signs of an upper respiratory infection, can harbor HRV in their bronchial tissue.

This phenomenon is significantly less frequent in nonasthmatic control subjects. Subjects with asthma involved in this study were not representative of an average population of patients with asthma because of the recruitment scheme, which was based on a clinical indication or qualification for a diagnostic bronchoscopy. Moreover, an overrepresentation of aspirin-sensitive asthma likely resulted from the interest of our center in this particular asthma phenotype. Although it is tempting to speculate that the presence of HRV in the lower airways is a feature and possible mechanism that contributes to asthma severity, this conclusion is premature, and subsequent longitudinal studies are needed to confirm the persistence of HRV in asthma and its relationship to characteristics of disease severity. Additional work is also needed both to determine the host-susceptibility factors for this apparent infection and to ascertain the mechanisms that may cause or contribute to greater disease severity. Nonetheless, these initial findings raise the possibility that rhinoviruses can have an additional role in asthma if they become a persistent infection, which then may contribute to the underlying severity of the disease.

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