

Rhinoviruses Infect the Lower Airways

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Rhinoviruses are the major cause of the common cold and a trigger of acute asthma exacerbations. Whether these exacerbations result from direct infection of the lower airway or from indirect mechanisms consequent on infection of the upper airway alone is currently unknown. Lower respiratory infection was investigated *in vitro* by exposing primary human bronchial epithelial cells to rhinoviruses and *in vivo* after experimental upper respiratory infection of human volunteers. Bronchial infection was confirmed by both approaches. Furthermore, rhinoviruses induced production of interleukin-6, -8, and -16 and RANTES and were cytotoxic to cultured respiratory epithelium. This evidence strongly supports a direct lower respiratory epithelial reaction as the initial event in the induction of rhinovirus-mediated asthma exacerbations. The frequency of infection and the nature of the inflammatory response observed are similar to those of the upper respiratory tract, suggesting that rhinovirus infections may be one of the most important causes of lower in addition to upper respiratory disease.

Each of us spends >1 year of life with common colds [1]. Rhinoviruses cause the majority of such colds [2] but have been thought to be associated only with mild self-limiting upper respiratory tract infections. However, recently rhinoviruses have been associated with asthma exacerbations in both children [3] and adults [4], indicating that infection with these agents may result in significant morbidity and even mortality [5]. Furthermore, rhinovirus infections have also been linked clinically with other serious lower airway illnesses, including cystic fibrosis, bronchitis, bronchiolitis, and pneumonia [6–9]. These data have stimulated considerable interest in the pathogenesis of rhino-

virus-induced lower respiratory tract pathology and especially asthma exacerbations [10, 11]. Crucial to our understanding of such mechanisms is the debate over whether asthma exacerbations are a result of direct infection of the lower airways by rhinovirus or of indirect mechanisms consequent on infection of the upper airway alone [12]. If the former case is true, induction of local immunologic and inflammatory responses could initiate a cascade of events ultimately leading to clinical disease. Furthermore, prevention of spread of rhinovirus from the upper into the lower airways would become an important therapeutic target.

Although no conclusive evidence exists that rhinoviruses can infect and be directly pathogenic to the lower airways, several indirect observations support this hypothesis. After experimental infection, the virus has been isolated from lower airway secretions sampled by bronchoscopy [13], rhinovirus RNA has been detected in bronchoalveolar lavage cells by polymerase chain reaction (PCR) [14], and rhinoviruses have been cultured from sputum [15]. However, in all of these studies, contamination from the upper airways could not be excluded.

The presence of inflammation in the lower airways of rhinovirus-infected subjects [16] can best be explained by lower airway infection and the release of locally acting proinflammatory mediators. Nevertheless, evidence against this hypothesis is also available. Propagation of rhinoviruses has been achieved only in a small number of cell lines, whereas rhinoviruses have been reported to replicate optimally at the temperature of the upper airway (33°C) and poorly at lower airway temperatures [17], and no cytopathic effect (CPE) has been observed in respiratory epithelial cells.

In support of lower airway infection by rhinoviruses, limited

Received 17 November 1999; revised 8 February 2000; electronically published 5 June 2000.

Presented in part: annual meeting of the American Thoracic Society, San Diego, 23–28 April, 1999 (abstract B29).

Informed consent was obtained from the subjects of this study, which was approved by the Southampton University and Hospitals Joint Ethics Subcommittee.

Grant support: Medical Research Council (grants G9328737 to P.G.B., S.T.H., and S.L.J. and G8604034 to S.T.H.), the National Asthma Campaign (grant 332 to S.L.J.), the Wessex Medical Trust (to P.J.B. and S.L.J.), the Royal Society (to P.J.B. and S.L.J.), and the University of Ferrara (to A.P.).

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The Journal of Infectious Diseases 2000;181:1875–84

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0022-1899/2000/18106-0001\$02.00

replication was recently documented in transformed cell lines of lower respiratory origin [18, 19]. In addition, we have recently demonstrated that, although most rhinovirus serotypes replicate optimally at 33°C, the higher temperature of the lower airways is not a preventive factor for their replication, and some strains may even prefer it [20].

We sought to describe replication of rhinoviruses in primary human bronchial epithelial cells (HBECs) and studied proinflammatory responses and CPE in these cells. More important, we used in situ hybridization to localize rhinovirus infection to the lower airways of human volunteers following an experimentally induced nasal infection.

Methods

Viruses. Rhinoviruses RV2 and RV7 were originally obtained from the Medical Research Council Common Cold Unit (Salisbury, UK), and their identity was confirmed by neutralization with specific antisera (American Type Culture Collection [ATCC], Rockville, MD). Viruses were propagated in large quantities in Ohio HeLa cells, at 33°C, in a humidified 5% CO₂ incubator (Forma Scientific, Marietta, OH). When full CPE developed, cells and supernatants were harvested, pooled, frozen and thawed twice, clarified, sterile-filtered, and aliquoted. Viruses were stored at -70°C. For each experiment, a new vial was rapidly thawed and used immediately. The RV16 used in experimental infection was a gift of W. Busse and E. Dick (University of Wisconsin, Madison).

Cell cultures. HBECs were obtained as described elsewhere [21]. Briefly, under a dissecting microscope, the submucous layer was removed from normal areas of bronchial tissue obtained from surgical resection material. The epithelial layer was cut into 1–2-mm squares, which were placed in 24-well plates (Falcon Primaria; Becton Dickinson, Cowley, UK) in medium 199 with 2% Ultrosor-G (Gibco BRL, Paisley, UK). Cells were allowed to outgrow until ~70% confluent. One day before the experiment, the explant was removed, each monolayer was examined microscopically, and wells with fibroblast contamination were discarded. In addition, immunocytochemical analysis for cytokeratins 13 and 18 and flow cytometry with a pan-cytokeratin antibody (Sigma, Poole, UK) confirmed that, in all cases, >95% of the cells were of epithelial origin.

HBEC infection. HBECs were exposed to rhinoviruses at an MOI of 1 for 1 h with gentle shaking, in parallel with control wells exposed to medium from noninfected Ohio HeLa cell cultures. The virus solution was removed, and cells were washed twice with PBS, replenished with fresh medium 199 with 2% Ultrosor-G (Gibco), and incubated at 33°C. Cell morphology was examined microscopically daily. Supernatants were removed at various times after infection and clarified by centrifugation. At the same times, cell monolayers were detached by scraping, resuspended in 1 mL of PBS, and snap-frozen and thawed twice. Both supernatants and cell lysates were stored at -70°C until assayed.

Effect of rhinovirus-inactivating factors. Rhinoviruses were incubated with a 1 : 10 dilution of specific antisera (aRV2 and aRV7, ATCC) or 1 mg/mL soluble intercellular adhesion molecule-1 (provided by P. Esmon, Bayer, Berkley, CA) for 45 min at room temperature. Furthermore, rhinoviruses were inactivated by exposure

to pH 3 for 1 h at 4°C [22]. These suspensions were used to infect HBEC monolayers in parallel with noninactivated controls. Supernatants and cell lysates were harvested at 24 and 48 h after infection and were used to assess the specificity of viral infection and cytokine production in titration assays and immunoassays, respectively.

Rhinovirus infection of human volunteers. The subjects described herein were previously investigated in a study by Fraenkel et al. [16]. Sufficient bronchial biopsy material from 10 subjects was available. Seven subjects were normal volunteers, and 3 had asthma (table 1). All were nonsmokers, were negative for or had low titers (<1 : 2) of neutralizing antibodies to RV16, and had not experienced any upper respiratory tract infection in the previous 6 weeks. Infection was done with a total of 5000–10,000 TCID₅₀ of RV16 on 2 subsequent days, by nasal aerosol insufflation with a DeVillbiss 286 atomizer (DeVillbiss, Somerset, PA; mass median aerodynamic diameter, >10 μm) and by nasal droplet instillation [16, 24–26]. Nasal aspirates were taken at baseline and on each of the subsequent 3 days after inoculation. Bronchial biopsy samples were taken at baseline and 3 days after inoculation, according to methods described elsewhere [3, 16, 25]. Subjective scores (0 [absent] to 3 [severe]) for cold symptoms and chest symptoms [16, 26] were recorded and accumulated over a 5-day period after inoculation. Then, 6–8 weeks later, the sampling was repeated, and a blood sample was taken to determine the increase in specific antibodies to RV16 [16, 26]. Biopsy specimens were fixed for 24 h in 10% neutral-buffered formalin and embedded in paraffin wax blocks.

Virus titrations. Rhinovirus titers of original virus stocks, HBEC lysates and supernatants, and nasal aspirates were determined according to standard protocols [20]. Briefly, Ohio HeLa cells were seeded 2 days before infection in 96-well plates, reaching 60%–70% confluence at the time of infection. Logarithmic dilutions of the samples to 1 : 10⁻⁹ were made in quadruplicate wells. After 7 days, plates were fixed and stained with 5% formaldehyde, 5%

Table 1. Subject profiles, infection scores, and results of in situ hybridization for rhinovirus.

Condition, subject	Sex/age	Culture score	Cold score	Chest score	In situ hybridization result ^a		
					Baseline	Cold	Convalescence
Normal							
1	M/50	12	5	2	ND	+ (4)	- (5)
2	M/49	12	6	1	- (2)	- (4)	- (3)
3	F/45	12	24 ^b	7	- (6)	+ (5)	++ (5)
4	F/45	12	7	1	- (4)	- (4)	- (2)
5	F/21	10	13	3	- (4)	- (4)	- (3)
6	F/20	4	10	3	- (2)	- (2)	+ (4)
7	M/20	12	36 ^b	6	- (4)	++ (2)	- (3)
Asthmatic							
8	M/26	12	18 ^b	7	- (6)	++ (3)	- (2)
9	M/27	12	24 ^b	1	- (7)	++ (7)	- (5)
10	M/22	5	24 ^b	10	- (2)	- (3)	- (3)

NOTE. M, male; F, female. Age is given in years. Culture scores represent cumulative virus titers in nasal aspirate samples after experimental infection [16]. Cold and chest scores are indexes of subjective symptoms from the upper and lower respiratory tracts [16].

^a ND, not determined; -, no signal; +, moderate signal; ++, strong signal. Total nos. of independent experiments with equivalent results are shown in parentheses.

^b Symptomatic colds according to criteria of Jackson et al. [23].

Table 2. Primer pairs, amplicon sizes, and thermal cycling conditions for cytokine and chemokine reverse transcription–polymerase chain reaction.

Cytokine or chemokine	Primer pair	Product size (bp)	Thermal cycles (denaturation, annealing, extension, × no. of cycles)
Interleukin-6	ATGAACTCCTTCTCCACAAGCGC GAAGAGCCCTCAGGCTGGACTG	628	94°C, 15 s; 50°C, 30 s; 72°C, 30 s, ×35
Interleukin-8	ATGACTTCCAAGCTGGCCGTGGCT TCTCAGCCCTCTTCAAAAAGTCTCTC	289	94°C, 15 s; 50°C, 30 s; 72°C, 30 s, ×28
Interleukin-16	ATGCCCCGACCTCAACTCCTC CTCCTGATGACAATCGTGAC	347	94°C, 20 s; 60°C, 30 s; 72°C, 40 s, ×40
RANTES	ATGAAGGTCTCCGCGGCACGCCTCGTGTC CTAGCTCATCTCCAAAGATTGAT	252	94°C, 15 s; 53°C, 20 s; 72°C, 30 s, ×38
Macrophage inhibitory protein-1 α	CAGGTCTCCACTGCTGCC CACTCAGCTCCAGTCACT	252	94°C, 15 s; 50°C, 20 s; 72°C, 30 s, ×40

ethanol, and 0.1% crystal violet in PBS, and the end-point titer, defined as the highest dilution at which a CPE was detected in half of the wells (TCID₅₀), was read and was expressed as the inverse logarithm of this dilution. Shedding of RV16 in nasal aspirates was quantified by a system previously devised [16, 26], in which a CPE was scored as 0 (absent) to 4 (maximum) and was expressed as a total of the 3 samples taken after inoculation. Results were confirmed by neutralization, using RV16-specific antisera (ATCC).

Immunoprecipitation of viral proteins. Newly synthesized viral proteins in HBECs exposed to RV7 were examined by immunoprecipitation [27]. The virus was inoculated on HBEC monolayers at an MOI of 1 for 1 h with gentle shaking. After 24 h of incubation at 33°C, the medium was replaced by methionine-deficient medium (Sigma). Thirty minutes later, [³⁵S]methionine (Amersham, Little Chalfont, UK) was added to a final concentration of 50 μ Ci/mL and the incubation was continued for another 24 h, after which the medium was discarded, cells were washed twice, and cell pellets were collected and suspended in 1 mL of lysis buffer (1% Triton X-100 in PBS). Lysates were stored in 0.25-mL aliquots at –80°C until further assayed. After thawing, an additional 0.75 mL of lysis buffer was added, and lysates were sonicated and incubated at 4°C for 10 min. To reduce nonspecific binding, 50 μ L of *Staphylococcus* protein A–Sepharose Fast Flow (Sigma) was added, lysates were agitated at 4°C for 1 h, protein A–Sepharose was pelleted in a microcentrifuge (Jouan Ltd., Ilkeston, UK) at 15,000 *g* for 1 min, and the supernatant was collected. A protein A–Sepharose–polyclonal antibody complex was prepared by adding 5 μ L of guinea pig polyclonal anti-RV7 antibodies (ATCC) to 100 μ L of *Staphylococcus* protein A–Sepharose Fast Flow and agitating for 2 h at room temperature. This complex was added to the cell lysate supernatant, and agitation was continued overnight at 4°C.

After this incubation, pellets were washed 3 times in PBS, 50 μ L of the sample buffer (24% glycerol, 5% SDS, 12% β -mercaptoethanol, 0.001 *M* bromophenol blue, and 1.5 *M* Tris-HCl, pH 6.8) was added, the solution was heated at 95°C for 2 min and centrifuged, and the protein A–Sepharose pellet was removed. The supernatant was loaded onto a 10% SDS-PAGE gel and electrophoresed at 30 mA. Gels were fixed in 40% methanol, 10% acetic acid, and 3% glycerol for 1 h, incubated in Amplify (Amersham) for 30 min, washed, dried, and autoradiographed.

Detection of rhinovirus RNA. Reverse transcription (RT)–PCR for rhinovirus RNA was done as described elsewhere [28], with minor modifications. RNA was extracted from cell lysates as follows: 100 μ L of Trizol reagent (Gibco) was added to an equal

volume of cell lysate in Eppendorf tubes. After 5 min at room temperature, 20 μ L of chloroform was added, and tubes were shaken vigorously for 15 s and further incubated for 2–3 min at room temperature. They were then centrifuged at 12,000 *g* for 15 min, the aqueous phase was removed, and the RNA was precipitated with isopropanol, washed with 75% ethanol, air-dried, and resuspended in 10 μ L of ultra-high-quality water. RT was done in a buffer consisting of 50 *mM* Tris-HCl, 75 *mM* KCl, and 3 *mM* MgCl₂, with 10 *mM* dithiothreitol, 0.4 *mM* dNTPs, 0.5 μ g of random hexamer primers (Promega, Southampton, UK), and 100 U of reverse transcriptase (Superscript; Gibco). The mix was incubated at 37°C for 60 min to yield cDNA.

PCR was done in a total volume of 50 μ L with buffer consisting of 10 μ L of cDNA, 10 *mM* Tris-HCl, 50 *mM* KCl, and 0.1% Triton X-100 with 1.5 *mM* Mg²⁺, 0.2 *mM* dNTPs, 4.25 U of Taq DNA polymerase (Promega), and 1.5 μ M primers OL27 (5'-CGGACA-CCCAAAGTAG-3') and OL26 (5'-GCACTTCTGTTTCCCC-3') (Oswel DNA Service, University of Southampton), which are complementary to the antisense RNA at positions 542–557 and 169–185 in the 5' noncoding region of RV1b. The thermal cycle consisted of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min for 32 cycles, including a post-PCR extension step at 72°C for 4 min. A 380-bp amplicon was generated, visualized by ethidium bromide staining after electrophoresis on a 2% agarose gel. The specificity of the PCR product was examined by restriction fragment length polymorphism analysis with *Bgl*I (Promega), as recently described elsewhere [29]. To confirm the identity of RV16 in biopsy samples, amplicons were sequenced on an automated sequencer (373A; Applied Biosystems, Warrington, UK), using the Taq Dye Deoxy Terminator Cycle (Applied Biosystems) protocol.

Cytokine and chemokine mRNA expression. Semiquantitative cytokine RT-PCR assays were done on cDNA from HBEC lysates, in a total volume of 50 μ L, with 1.5 *mM* Mg²⁺, 0.2 *mM* dNTPs, 2.5 U of Taq DNA polymerase (Qiagen, Crawley, UK), and 0.5 μ M primers. Interleukin (IL)–6, IL-8, IL-16, RANTES, and macrophage inhibitory protein-1 α (MIP-1 α) were examined. Primer pairs and thermal cycling conditions are shown in table 2. Parallel PCR amplification of β -actin mRNA was used as a control of cDNA loading. Densities of PCR bands were measured by Band Leader software, version 3.00 (TechKnowledge, Tel Aviv, Israel), and were expressed as a percentage of their respective β -actin value.

Measurement of cytokine and chemokine production. Levels of IL-6, IL-8, RANTES, and MIP-1 α were measured in culture su-

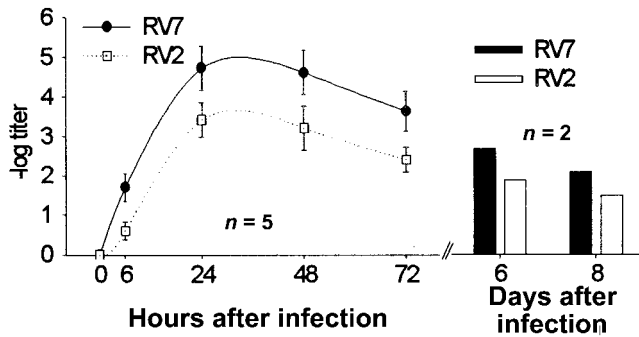


Figure 1. Time-dependent increase in virus titers in human bronchial epithelial cells exposed to rhinoviruses RV2 and RV7. Although no virus was detectable immediately after infection, significant titers were observed thereafter, peaking at 24–48 h after infection. Differences between 0 and 6 h and between 6 and 24 h are significant ($P < .05$; $n = 5$). Virus titers gradually declined; however, live virus was still present intracellularly 8 days after infection.

pernatants by use of a fluoroimmunoassay. Low-fluorescence immunoassay plates (Fluoronunc; Nunc, Roskilde, Denmark) were coated overnight at room temperature with the appropriate antibody solution (R&D Systems, Abingdon, UK) at a concentration of 4 $\mu\text{g}/\text{mL}$. After nonspecific binding sites were blocked with 1% bovine serum albumin, 5% sucrose in PBS (Sigma), standards, and appropriately diluted samples were added and incubated at 4°C overnight, followed by incubation with a biotinylated secondary antibody for 1 h at 37°C. Biotinylated antibody concentrations were 25 ng/mL for IL-6, 20 ng/mL for IL-8, 5 ng/mL for RANTES, and 40 ng/mL for MIP-1 α (R&D Systems). Positive samples were detected by incubation with a europium-streptavidin conjugate (DELFLIA; EG&G, Milton Keynes, UK) for 1 h at 37°C, followed by DELFLIA enhancement solution (EG&G) for 15 min at room temperature with gentle shaking. This solution dissociates europium ions from the solid phase into solution, to form highly fluorescent chelates with ligands present in the solution. Europium-mediated fluorescence was measured with a fluorometer (DELFLIA 1234; Wallac, Turku, Finland). The assay volume was 80 μL in all cases. Wells were washed 4 times before proceeding to the next step. The sensitivity of the assays was as follows: IL-6, 2 pg/mL; IL-8, 4 pg/mL; RANTES, 4 pg/mL; and MIP-1 α , 8 pg/mL.

A commercially available kit was used for the measurement of IL-16 (Biosource, Camarillo, CA), according to the manufacturer's instructions. The sensitivity of this assay was 5 pg/mL.

In situ hybridization. Antisense oligonucleotide probes for RV16, targeting sequences of the 5' noncoding region, have been described elsewhere [30]. These were as follows: PB4, CAGGGG-CCGGAGGACTCAAGATGAGCACACGCGGCTC; PB5, TG-CAGGCAGCCACGCAGGCTAGAACTCCGTCGCCG. Two further oligonucleotide probes were designed, also complementary to regions of the 5' noncoding region of RV16: PB6, ACA-CGGACACCCAAAGTAGTTGGTCCCATCCCAGCA; PB7, ACATCCTTAACTGGGTCTGTGAATTTACTGGGGTCT. A cocktail of the 4 probes was used. Oligonucleotides were synthesized and purified by Oswel DNA Services (University of Southampton, Southampton, UK) and labeled with digoxigenin-dUTP

by use of an oligonucleotide 3' tailing kit (Boehringer Mannheim, Lewes, UK). A single random nonsense sequence with a length, GC content, and melting temperature similar to those of the rhinovirus probes [30] was used as the negative control, because if the virus is replicating, both sense and antisense strands should be present. Replicative-strand rhinovirus RNA was detected by use of sense probes complementary to PB4–PB7.

In situ hybridization was done on 4- μm biopsy sections, as described elsewhere [30]. Briefly, sections were initially dewaxed by immersions in xylene and a graduated series of rinses in ethanol and water and then were treated with 5 $\mu\text{g}/\text{mL}$ proteinase K for 60 min at 37°C to permeabilize the tissue. Hybridization took place in a humidity chamber at 37°C overnight, in $\times 2$ buffer (Amersham), 30% (v/v) deionized formamide, and 10 $\mu\text{g}/\text{mL}$ poly(dA)_(25–30) (Sigma) as an additional blocking agent, with a final concentration of each probe of 50 ng/mL. After 3 posthybridization washes, an anti-digoxigenin alkaline phosphatase-conjugated antibody (Boehringer Mannheim) was added for 60 min at room temperature. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim) were used as alkaline phosphatase substrates. The development of cellular coloration (blue-black) was monitored for up to 8 h by low-power ($\times 100$) light microscopy and was stopped, when necessary, by washing the slides in water. Sections were coded and analyzed blindly on 2–7 separate occasions. Sections from baseline, cold, and convalescent biopsy samples from the same subject were run together. A positive (poly[dT]) and negative (nonsense) control probe were always included.

Statistical analysis. Data are expressed as mean \pm SE. Testing

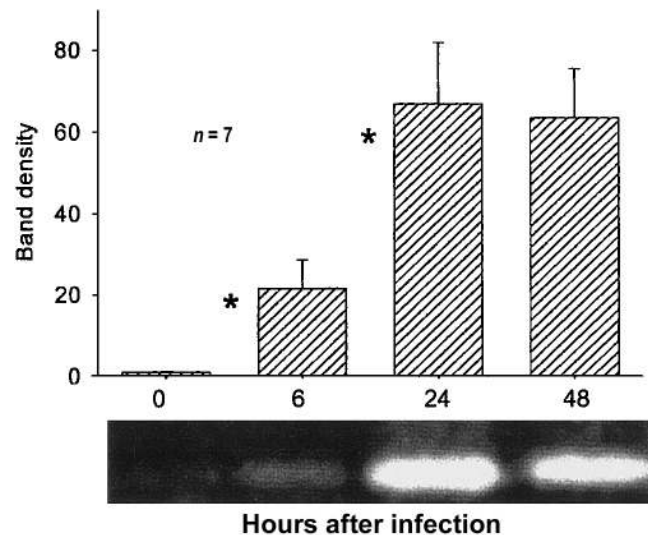


Figure 2. Presence of rhinovirus (RV) RNA in human bronchial epithelial cells assessed by reverse transcription-polymerase chain reaction 0, 6, 24, and 48 h after infection. Ethidium bromide-stained bands were scanned and quantified by densitometry. Significant increases between 0 and 6 h and between 6 and 24 h were observed, in a time course similar to that of virus titers. Bars, mean \pm SE of 7 experiments from both RV2 and RV7 infections. Representative gel is shown under the graph. * $P < .05$ vs. previous time point.

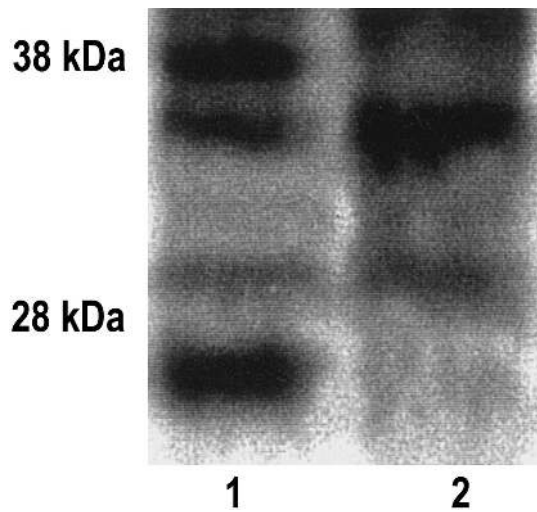


Figure 3. Autoradiograph of SDS-PAGE of newly synthesized [^{35}S]-labeled rhinovirus (RV)-7 proteins immunoprecipitated with specific polyclonal antiserum. Viral capsid proteins VP1 (38 kDa) and VP3 (28 kDa) are seen in *lane 1* (RV7-infected cells) but not in *lane 2* (negative control).

for statistical significance in the time course studies was done by analysis of variance, followed by Student's paired *t* tests at each time point. Other comparisons of means were done by the Wilcoxon matched pair test.

Results

Infection of HBECs with rhinovirus. To investigate whether rhinoviruses can infect primary human lower airway epithelium, subconfluent monolayers of HBECs were exposed to RV2 (minor group rhinovirus) and RV7 (major group rhinovirus) for 1 h at an MOI of 1 and then were washed. No cell-associated virus was detectable by titration assay of cell lysates immediately after the inoculation, whereas the virus preparation used for the inoculation retained its original titer, indicating that only a very small number of viruses had entered the cells. Low titers became detectable 6 h after infection, progressively increasing until 24–48 h, followed by a gradual decline (figure 1). An increase in titers was also observed in clarified supernatants between 6 and 48 h after infection (not shown).

An identical pattern was observed when intracellular rhinovirus RNA levels were determined by RT-PCR. Very faint or no PCR bands were observed 2 h after infection, significantly increasing over 6 and 24 h (figure 2). Peak viral RNA production was observed at 24 h in 5 of 7 experiments and at 48 h in the remainder. Experiments done 4, 6, and 8 days after infection also gave positive PCR signals, with a gradually declining trend similar in time course to that of virus titers.

Synthesis of new rhinovirus proteins in HBECs was investigated by immunoprecipitation of [^{35}S]-labeled viral proteins with specific antiserum. Although the sensitivity of this method

is low with the available number of cells, newly synthesized rhinovirus capsid proteins, principally VP1 (38 kDa) and VP3 (28 kDa), were clearly present 48 h after infection (figure 3).

Preincubation of RV2 and RV7 with their respective specific polyclonal antisera was able to completely block infection. Furthermore, preincubation with soluble ICAM-1, the receptor of major group rhinoviruses [31], was able to decrease the titer achieved by RV7 by 1000-fold but had no effect on RV2 (figure 4).

Development of CPE. HBEC viability was consistently >90%, as assessed by trypan blue exclusion, in all cultures described above. To examine whether the absence of cytotoxicity on infected HBECs was related to the amount of infecting virus, infections were done at higher concentrations of the virus (MOI of 10–30) and at lower confluence. Although increased numbers of floating cells were apparent in confluent cultures, no CPE was observed, and the monolayers remained intact. However, when the same relative amount of virus was inoculated on wells with low confluence, an intense and typical rhinovirus CPE was observed after 48 h (figure 5).

Cytokine mRNA expression and protein production. An inflammatory response, in which cytokines and chemokines are crucial mediators, is central to the development of asthma. Experiments were therefore done to examine the induction of cytokines and chemokines important in the pathogenesis of asthma in rhinovirus-infected HBECs. Noninfected HBECs expressed mRNA for IL-6, IL-8, IL-16, RANTES, and MIP-1 α . Infection with RV7 resulted in a significant increase over baseline expression after 24 h for IL-6, IL-8, IL-16, and RANTES.

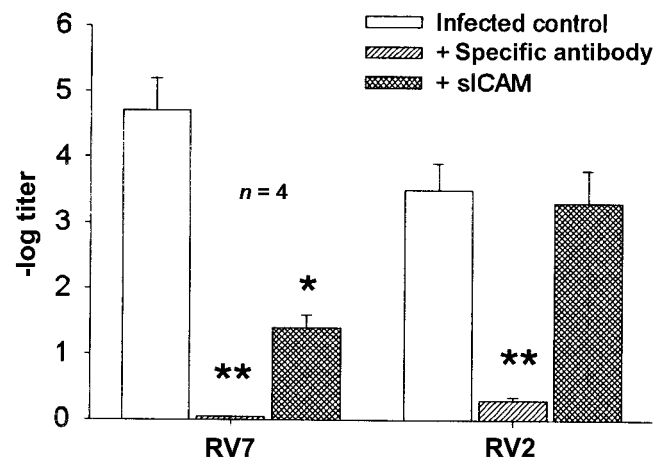


Figure 4. Specificity of rhinovirus (RV) infection in human bronchial epithelial cells assessed by preincubating virus suspensions with either specific polyclonal antibodies or soluble intercellular adhesion molecule-1 (sICAM-1). Preparations were subsequently used to infect cells in parallel with nontreated positive controls. Specific antibody pretreatment was able to prevent infection in both cases. sICAM-1 reduced RV7 (major serotype) titers by >1000-fold but had no effect on RV2 (minor serotype). **P* < .05 vs. control; ***P* < .01 vs. control.

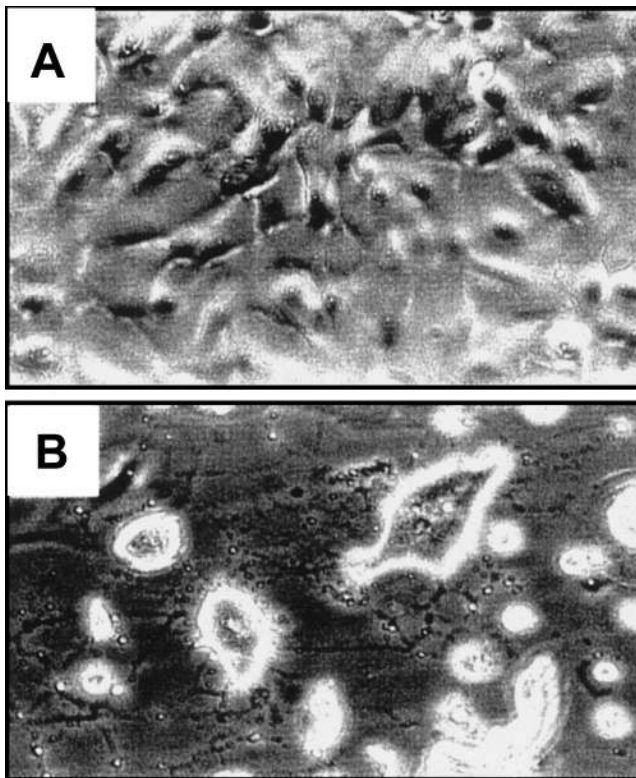


Figure 5. Normal human bronchial epithelial cell (HBEC) monolayer (A), shown in comparison with characteristic rhinovirus (RV) cytopathic effect produced 48 h after infection of sparse culture of HBECs with 10 MOI of RV7 (B).

At 48 h after infection, mRNA expression returned to baseline values for IL-6 and IL-16, increased further for RANTES, and remained at the same level for IL-8 (figure 6). No increase in mRNA was observed for MIP-1 α (not shown).

To establish that these cytokines and chemokines are secreted, protein levels in supernatants of infected and noninfected cells were measured at 48 h after infection. Consistent with mRNA expression, there was significant induction of IL-6, IL-8, IL-16, and RANTES protein production by RV7-infected cells (figure 7). No MIP-1 α production was detectable. Receptor blockade and acid inactivation, both of which inhibited rhinovirus-induced IL-8 and RANTES production (figure 8), confirmed rhinovirus specificity of the induction.

Experimental rhinovirus infection in human volunteers. To confirm the *in vivo* relevance of our *in vitro* findings, experimental rhinovirus colds were induced in 7 normal and 3 asthmatic human volunteers. Intranasal inoculation of RV16 resulted in upper respiratory infection in all 10 volunteers, assessed by detection of virus in nasal aspirates 3 days after inoculation (table 1). Five of the subjects also seroconverted. A clinical cold, as indicated by a cold symptom score ≥ 14 [23], was induced in 5 of the 10 subjects (table 1). The remaining 5 subjects reported cold scores of 5–13, indicating that milder

symptoms were present. The 3 asthmatic subjects reported high cold symptom scores of 18–24; however, one of them (subject 10) had a relatively small increase in titer and low cell culture score, suggesting a low-level infection despite high severity of symptoms.

Detection of rhinovirus in the lower airways after experimental infection. The presence of rhinovirus in the lower airways after upper respiratory experimental infection was investigated by *in situ* hybridization for RV16 on bronchial biopsy samples taken before infection (baseline), during the peak of the infection, and 6–8 weeks after infection (convalescent). All baseline biopsy samples were negative. Positive signals for RV16 were consistently detected in the bronchial biopsy samples of 5 of 10 subjects during infection (table 1; figure 9). No signal was observed in sections taken from the infected biopsy samples when hybridized with the control probes at any time.

RV16 in bronchial biopsy tissue was predominantly localized to the columnar epithelial layer but also included the basal cell layer. Infection was also detected occasionally in subepithelial cells but could not be identified as being associated with any specific cell type. The use of a counterstain (nuclear red, not shown), suggested that the hybridization signal in bronchial epithelium involved principally the perinuclear region of positive cells, but with 2-dimensional imaging it was difficult to be more precise with regard to cellular localization.

Positive signal was also detected in convalescent biopsy samples from 2 subjects (subjects 3 and 6). Subject 3 reported having

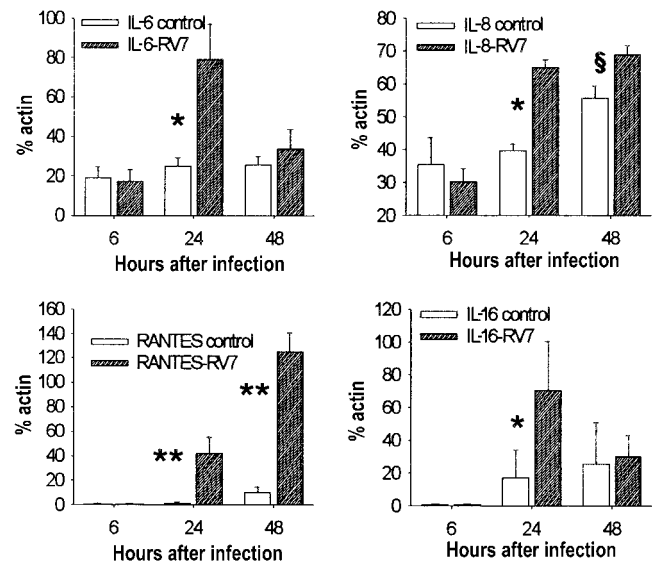


Figure 6. Time courses of interleukin (IL)-6, IL-8, RANTES, and IL-16 mRNA expression in rhinovirus RV7-infected and control human bronchial epithelial cells, assayed in parallel. Reverse transcription-polymerase chain reaction bands were quantified by densitometry and were expressed as percentage of their respective β -actin (housekeeping gene) value. Data are mean \pm SE of 6–11 experiments. * $P < .05$ for control vs. infected; ** $P < .01$; $^{\S} .05 < P < .1$.

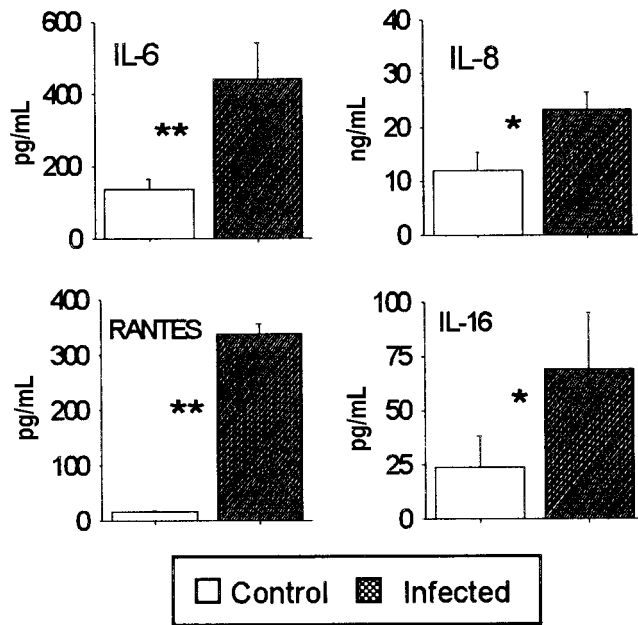


Figure 7. Levels of interleukin (IL)-6, IL-8, RANTES, and IL-16 in supernatants collected 48 h after infection of human bronchial epithelial cells with 1 MOI of rhinovirus RV7, compared with noninfected control cultures run in parallel. Data are mean \pm SE ($n = 8-11$). * $P < .05$; ** $P < .01$.

a naturally occurring cold when the convalescent samples were obtained. Upper respiratory rhinovirus infection in this subject was confirmed by cell culture of the nasal aspirate sample and by acid lability testing, indicating that the subject was infected with a wild-type rhinovirus at this phase of the study. A nasal aspirate was not taken for subject 6 because this subject reported no symptoms.

Confirmation of positive in situ hybridization signals by sequencing. To validate the above findings, RT-PCR for rhinovirus was done on RNA extracted from sections cut from 3 of the biopsy samples positive by in situ hybridization, followed by sequencing of the PCR products. In all 3 cases, a strong signal was obtained by PCR, and the sequence of the amplicons was identical to the published sequence for RV16 in this region [32].

Detection of rhinovirus replication by in situ hybridization for replicative-strand RNA. Having confirmed the presence of rhinovirus in the lower airway epithelium by in situ hybridization, we wished to investigate whether this was accompanied by signals indicating active viral replication. Detection of rhinovirus replication was investigated by use of complementary sense probes for replicative-strand rhinovirus RNA in 8 samples, 5 positive and 3 negative for genomic-strand rhinovirus RNA by in situ hybridization. In each of the 5 cases in which RV16 genomic strand was detected in the epithelium, strong signals for replicative-strand RNA were also observed. Furthermore, the signals obtained with antisense and sense probes

were colocalized in all instances. No signals were observed in the samples negative by in situ hybridization for genomic RNA when they were hybridized with probes for replicative-strand RNA.

Discussion

The ability of rhinoviruses to infect the lower airway is of critical importance in the pathogenesis of virus-induced asthma exacerbations, bronchitis, bronchiolitis, cystic fibrosis, and pneumonia and remains controversial [12, 33]. The data presented here confirm that, as well as being the most important upper respiratory tract pathogen, rhinovirus is also a lower

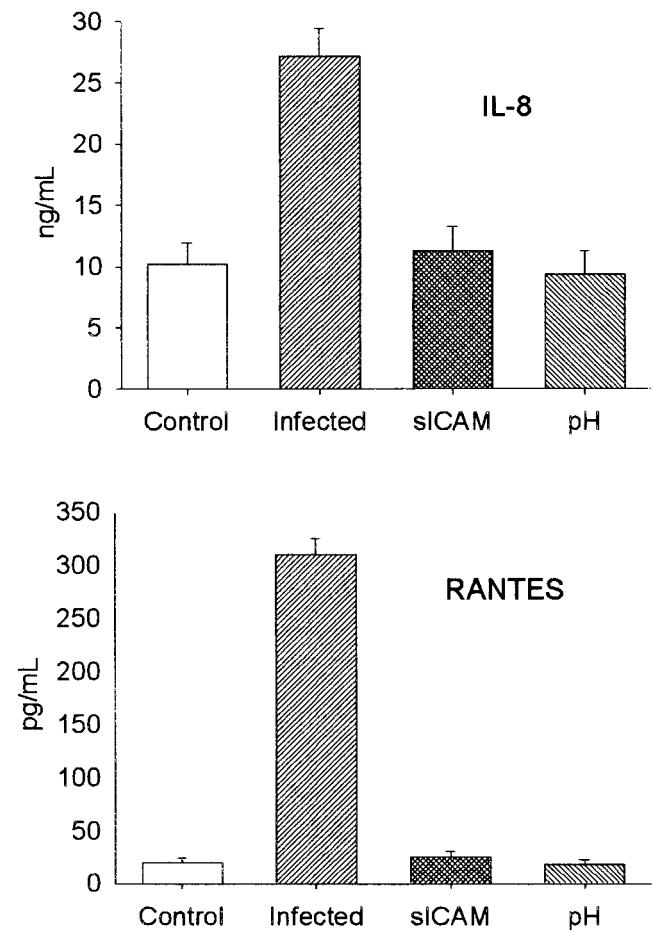


Figure 8. Effects of preincubation of rhinovirus RV7 with soluble intercellular adhesion molecule-1 (sICAM-1) or inactivation by exposure to pH 3 for 1 h on interleukin (IL)-8 and RANTES production. When human bronchial epithelial cells were infected with these preparations in parallel with noninfected (negative control) and nontreated virus-infected cells (second bars from left), IL-8 and RANTES production 48 h after infection was similar to that of noninfected cells, verifying specificity of mediator induction. Differences between inactivated and nontreated virus preparations are significant in all cases (IL-8, $P < .05$; RANTES, $P < .001$; $n = 4$).

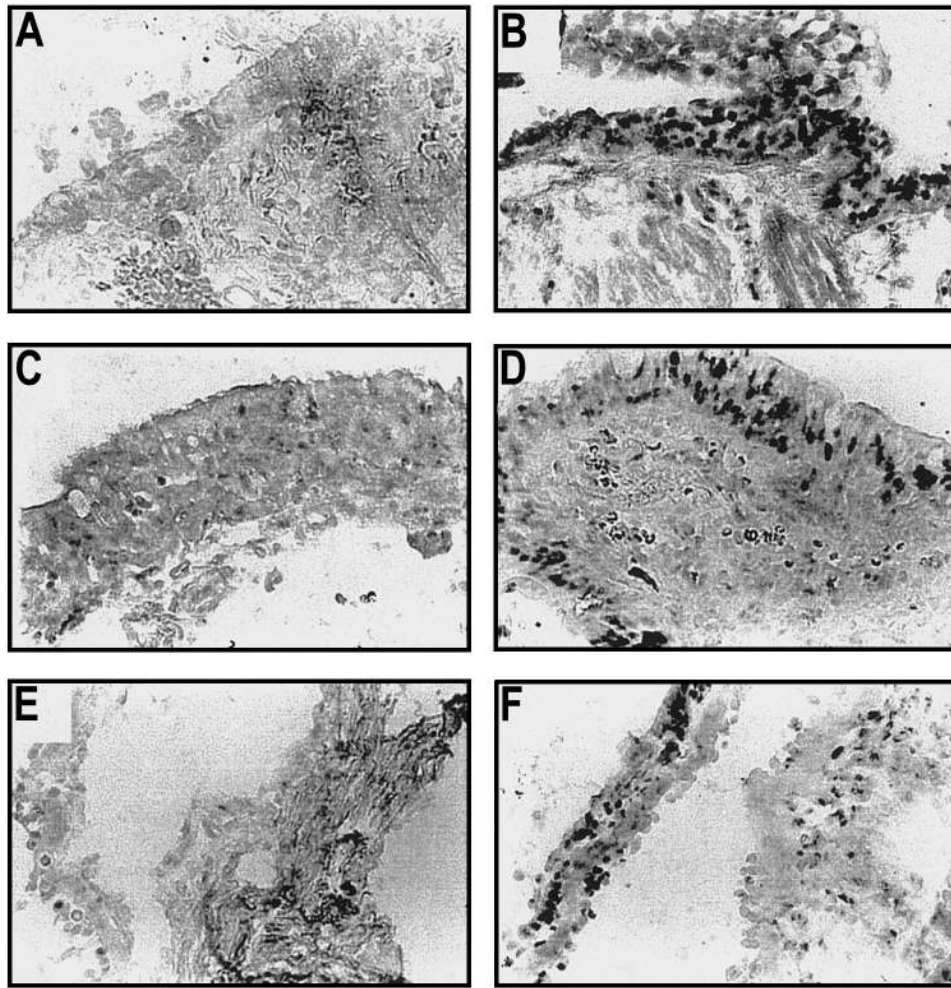


Figure 9. In situ hybridization for rhinovirus RV16 in sections of human bronchial biopsy samples. Negative bronchial biopsy samples taken before infection from subjects 3 (*A*), 7 (*C*), and 8 (*E*) are compared with RV16-positive biopsy samples from the respective subjects obtained during experimental RV16 infection (*B*, *D*, and *F*). The hybridization signal for RV16 is visible as black color in the cells and is localized mainly on epithelium. Magnification, $\times 400$.

respiratory tract pathogen. We have demonstrated conclusively that rhinovirus can reach, penetrate, and replicate in lower airway epithelium of both normal and asthmatic persons after nasal inoculation. The implementation of in situ hybridization excludes the possibility of sample contamination by rhinovirus derived from the upper airways, which has been the major drawback of previous attempts to investigate the ability of rhinovirus to replicate in the lungs [13, 14]. Rhinovirus replication in lower airway epithelium was confirmed in vitro by time-dependent increases in virus titers and viral RNA and production of new viral proteins, as well as in vivo with in situ hybridization probes complementary to the replicative strand of the virus.

The characteristics of rhinovirus infection of bronchial epithelium are similar to those reported in the upper respiratory tract. In vitro, HBECs initially take up a very small amount

of virus, and virus production increases significantly over the next 24–48 h, followed by a gradual decline. This was also the case in nasal [34] and tracheal [35] epithelium, as well as in a model of rhinovirus replication in BEAS-2B cells, an adenovirus-transformed cell line of bronchial epithelial origin [19].

The rhinovirus identification rate in vivo (50%) is almost identical to those in similar studies that used in situ hybridization on nasal biopsy samples, where rhinovirus infection is not disputed. In 2 such studies, the reported identification rates in either experimental or natural rhinovirus colds were 49% and 50%, respectively [30, 36]. Considering the fact that rhinovirus infections may be patchy in distribution [37] and the small size of the bronchial biopsy samples, the above data suggest that the frequency of rhinovirus infection of the lower airway is similar to that observed in the upper airway infections and that lower airway infection is probably a part of the natural

history of most common colds due to rhinoviruses. Furthermore, patients with positive in situ hybridization signals had a higher average rhinovirus culture score than did those negative by in situ hybridization (table 1), suggesting that lower airway infection with rhinovirus may be more likely in those with high nasal titers of rhinovirus. However, further studies are required to confirm this observation.

Spread of rhinoviruses in the lower airways during the experimental cold may have resulted from self-inoculation during respiration, coughing, or sneezing. Direct deposition of virus in the lower airways during the experimental inoculation is also possible but unlikely because of the large droplet size of the insufflation device (mass median aerodynamic diameter, >10 μm). Indeed, natural infections occur after inhalation of particles of all sizes, including those <5 μm , which are more likely to inoculate directly into the lower respiratory tract. The identification of positive bronchial biopsy samples after 1 proven and 1 probable wild-type rhinovirus infection also suggests that involvement of the lower airways occurs after natural rhinovirus infections.

Another interesting finding of this study is the ability of rhinoviruses to produce CPE in HBECs. The viability of confluent or subconfluent monolayers was not affected by rhinovirus infection, consistent with previous studies of the effect of rhinoviruses on epithelial cell viability [19, 34, 35]. A CPE was, however, achieved when sparse cultures of HBECs were exposed to higher titers of virus. This is probably a function of cell density as well as virus titer, because the same concentration of virus did not affect confluent monolayers, whereas an MOI of 1 did not induce a CPE under any conditions. In addition, in a recent report, Schroth et al. [38] observed CPEs after infection of bronchial epithelial cells with RV49 but not RV16. It is not unlikely that differences between rhinovirus serotypes may also occur. Unlike other respiratory viruses, such as influenza virus, which can produce extensive epithelial damage in both the upper and lower airways, rhinovirus infection in vivo produces very little or no histologic alteration [39]. However, even if rhinovirus-induced cytotoxicity is difficult to detect clinically, it can clearly occur in vitro.

A further mechanism by which rhinovirus lower airway infection may lead to lower airway inflammation is the local induction of proinflammatory mediators. Production of the proinflammatory cytokines IL-6 and IL-8 has been reported after rhinovirus infection of epithelial cell lines [18, 19] and tracheal epithelium [35] in vitro, as well as in response to respiratory syncytial virus [40, 41] or influenza virus [42]. Induction of IL-8 has also been recently reported in bronchial epithelial cells [38] and is confirmed in this study. This is also the case for RANTES, a C-C chemokine with chemoattractant activity on eosinophils and T lymphocytes, which was strongly induced by rhinovirus infection of HBECs. We have recently reported increased concentrations of RANTES in nasal aspirates from children with natural colds, most of which were

caused by rhinoviruses [43]. The above studies are the first to describe rhinovirus-mediated induction of RANTES in vitro and in vivo. Similar up-regulation of RANTES has been reported in HBECs and cells lines infected with respiratory syncytial virus and influenza A virus [44, 45], suggesting that RANTES, IL-6, and IL-8 may contribute to a common lower respiratory epithelial response to respiratory viral infections.

IL-16 is a potent lymphocyte chemoattractant and activator, also acting on macrophages and eosinophils. Induction of IL-16 by respiratory viral infection has not been reported previously. Recent reports have indicated that IL-16 is the predominant lymphocyte chemoattractant in the pathogenesis of asthma [46, 47]. IL-16 is, therefore, a prime candidate in regulating the intense bronchial lymphocyte infiltration observed in experimental rhinovirus infection [16] and thus may be a critical mediator in rhinovirus-induced lower airway inflammation.

This study demonstrates that rhinoviruses are able to infect the lower airway and induce a proinflammatory response in both normal and asthmatic subjects. The frequency of infection and the nature of the inflammatory response are very similar to those observed using similar techniques in the upper airway. These data suggest that lower respiratory tract infection with rhinoviruses is likely to be the norm rather than the exception and that rhinovirus should now be considered a lower as well as an upper respiratory tract pathogen. Further studies investigating the clinical importance of rhinovirus infections in asthma, cystic fibrosis, bronchitis, bronchiolitis, and pneumonia are now required.

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