Low Grade Rhinovirus Infection Induces a Prolonged Release of IL-8 in Pulmonary Epithelium[1]

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Rhinoviruses are important respiratory pathogens implicated in asthma exacerbations. The mechanisms by which rhinoviruses trigger inflammatory responses in the lower airway are poorly understood, in particular their ability to infect the lower airway. Bronchial inflammatory cell (lymphocyte and eosinophil) recruitment has been demonstrated. IL-8 is a potent proinflammatory chemokine that is chemotactic for neutrophils, lymphocytes, eosinophils, and monocytes and may be important in the pathogenesis of virus-induced asthma. Increased levels of IL-8 have been found in nasal samples in natural and experimental rhinovirus infections. In these studies we therefore examine the ability of rhinovirus to infect a transformed lower airway epithelial cell line (A549) and to induce IL-8 protein release and mRNA induction. We observed that rhinovirus type 9 is able to undergo full viral replication in A549 cells, and peak viral titers were found 24 h after inoculation. Rhinovirus infection induced a dose- and time-dependent IL-8 release up to 5 days after infection and an increase in IL-8 mRNA expression that was maximal between 3 and 24 h after infection. UV inactivation of the virus completely inhibited replication, but only reduced IL-8 protein production and mRNA induction by half, while prevention of virus-receptor binding completely inhibited virus-induced IL-8 release, suggesting that part of the observed effects was due to viral replication and part was due to virus-receptor binding. These studies demonstrate that rhinoviruses are capable of infecting a pulmonary epithelial cell line and inducing IL-8 release. These findings may be important in understanding the pathogenesis of rhinovirus-induced asthma exacerbations. *The Journal of Immunology,* **1998, 160: 6172–6181.**

Rhinoviruses are the most common upper respiratory pathogens, causing the majority of common colds and the complications of otitis media, sinusitis, and bronchitis. Recent studies have also highlighted the importance of this virus type in the induction of exacerbations of asthma. Three recent studies have provided evidence that rhinoviruses are associated with the majority of exacerbations of asthma in both adults and children, both in the community (1, 2) and in more severe exacerbations requiring hospital admission (3).

The biology of rhinovirus infections remains poorly understood. Rhinoviruses infect nasal epithelial cells (4) and cause a local inflammatory response associated with the production of kinins (5) and, in atopic subjects, histamine (6). The nature of the nasal mucosal cellular immune response to rhinoviral infection is poorly defined (7), although increased numbers of neutrophils, eosinophils, and lymphocytes have been demonstrated in nasal secretions in rhinovirus infections (8, 9). The cytokines implicated in the recruitment and activation of these inflammatory cells in rhinovirus infections are largely unknown. Increased levels of IL-1, -6, and -8 have been demonstrated in nasal washings taken from subjects with experimental rhinovirus infection (10–12), IL-11 has been detected in nasal aspirate samples from infants and young children with symptoms of upper respiratory tract infection, some of whom had wheeze (13), and we have also recently demonstrated increased levels of IL-8 and the neutrophil product myeloperoxidase in nasal aspirates from children with wild-type rhinovirus infections (14). IL-8 is a potent neutrophil chemoattractant and activator. An important role for IL 8 in controlling neutrophil-mediated inflammation in rhinovirus infection was suggested by the finding of close correlations between the nasal aspirate IL-8 and myeloperoxidase levels and between myeloperoxidase levels and upper respiratory symptom severity (14).

Much less is known about the lower airway response to rhinoviral infection in normal subjects; indeed, there has been considerable debate over whether rhinoviruses can infect the lower airway (15). Two recent studies have investigated the lower airway in normal subjects during experimental rhinovirus nasal infections. The first found evidence of rhinovirus replication in lower airway lavage cell populations (16), and the second found a marked CD3^{+}, CD4^{+}, and CD8^{+} lymphocyte and eosinophil infiltration in bronchial biopsies (17).

Eosinophil bronchial mucosal infiltration is a cardinal feature of asthma pathogenesis (18), and eosinophil infiltration has also been implicated in virus-induced asthma exacerbations. Experimental infection studies have demonstrated that in asthmatic or allergic rhinitic subjects, the eosinophil infiltrate provoked by rhinovirus infection alone or in association with concurrent allergen challenge is more prolonged than that in normal subjects (17, 19).

The precise mechanisms by which rhinovirus infections trigger exacerbations of asthma are not known. However, an important

role for IL-8 is suggested by the recent demonstration of a correlation between IL-8 nasal lavage levels and increases in bronchial hyper-reactivity induced by rhinovirus experimental infections (12). Furthermore, IL-8 has been implicated in lymphocyte chemotaxis in vitro (20) and in eosinophil chemoattraction and activation in vivo and in vitro (21, 22).

Given the putative important role of IL-8 in virus-induced asthma exacerbations and the recent demonstration that rhinovirus infection stimulates the release of IL-8 from the transformed bronchial epithelial cell line BEAS 2B (23), we have evaluated the ability of rhinovirus to infect a pulmonary epithelial cell line (A549) and have investigated the mechanisms of release of IL-8. We found low grade productive, but noncytolytic, rhinovirus infection of the epithelium that caused a prolonged release of IL-8 protein and up-regulation of IL-8 mRNA. The induction of IL-8 was only partially dependent on viral replication.

Materials and methods

Cell culture

A549 cells, a transformed alveolar epithelial cell line (24), were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were split twice weekly. Ohio HeLa cells were obtained from the Medical Research Council Common Cold Unit (Salisbury, U.K.) and were split weekly. Both cell lines were cultured at 37°C in 5% carbon dioxide in Eagle's MEM. All tissue culture media (Cancer Center, University of Iowa, Iowa City, IA) contained 10% heat-inactivated FBS (HyClone, Logan, UT), 4 mM L-glutamine, and 80 μg/ml of gentamicin.

Viruses

Rhinovirus serotype 9 was obtained from the Medical Research Council Common Cold Unit, and its identity was confirmed by neutralization with specific antiserum (ATCC) (25). Rhinovirus type 9 was used for all studies described and was propagated in large quantities in Ohio HeLa cells. After development of a full cytopathic effect, the virus was harvested, clarified, aliquoted, and frozen at -70°C. The viral titer, determined as described below on a thawed vial of the stored frozen aliquots, was 2×10^6 tissue culture infective doses 50% ($TCID_{50}$)[4]/ml. For all experiments, a new vial of virus was rapidly thawed at 37°C and was used immediately at a multiplicity of infection (MOI) of 1, except where indicated.

Virus titrations

Rhinovirus titers were determined in Ohio HeLa cell monolayers in 96-well microtiter plate assays; 125 μl of 5% MEM was added to each well, followed by 100 μl of HeLa cells in 5% MEM at 3×10^5/ml. The plates were cultured in 5% CO_2 at 37°C. When confluent (24–48 h), 25 μl of sample or 10-fold dilutions thereof (eight replicates) were added to each well, and the plates were cultured for 5 days. The cytopathic effect (CPE) was determined by visual assessment and by assessment of the continuity of the monolayer after fixation in methanol and staining with 0.1% crystal violet. $TCID_{50}$ values were computed as previously described (25).

Measurement of IL-8 protein

A549 cells were cultured at 2×10^6/ml in 2 ml of medium in 12-well plates. After 24 h (when 80% confluent), virus at an MOI of 1 or control medium was added, and incubation was continued for 6, 24, 48, 72, 96, and 120 h. Dose-response studies were conducted using 10-fold dilutions of virus stock and were harvested at 24 h. The effect of UV- and soluble ICAM-1 (sICAM)-inactivated virus was studied at 24 and 120 h. Supernatants were harvested and stored in aliquots at -70°C. IL-8 protein was measured by specific ELISA (R&D, Minneapolis, MN). The sensitivity of the assay was 4.7 pg/ml.

Measurement of IL-8 mRNA by Northern analysis

A549 cells were cultured for 18 h in 100-mm plates, and then virus was added at an MOI of 1. Cells were harvested at 1, 3, 6, 24, 48, and 96 h. Experiments were repeated between three and five times for time points up to 24 h and twice for the 48 and 96 h points. Whole cell RNA was extracted

using RNAstat (Tel-Test B, Friendswood, TX) according to the manufacturer's instructions. RNA was resuspended in 25 to 50 μl of water and quantitated by absorbance at 260 nm.

The RNA (10 μg in each lane) was fractionated on a 1.5% denaturing agarose gel containing 2.2 M formaldehyde (26). *Escherichia coli* 23S and 16S ribosomal RNA were run as m.w. markers. Equal RNA loading was confirmed by ethidium bromide staining of 28S and 18S ribosomal RNA. A 350-bp IL-8 cDNA probe complementary to part of the IL-8 mRNA 5′ noncoding region was generated by an *Eco*RI digest of pGEM3Z, provided by Joost J. Oppenheim (National Cancer Institute, Frederick, MD). Probe (100 ng) was labeled using the BRL (Gaithersburg, MD) random primer DNA labeling system, and 10 μl of [α-^{32}P]CTP (DuPont-New England Nuclear, Boston, MA) following the manufacturer's instructions. Unbound nucleotides were removed using a Sephadex G-50 column. The RNA was transferred to GeneScreen Plus (New England Nuclear, Boston, MA) as recommended by the manufacturer and then UV cross-linked to the nylon membrane. Membranes were prehybridized for 6 h in 50% formamide, 1 M NaCl, 10% dextran, 1% SDS, 0.05 M Tris, and 1$\times$ Denhardt's solution. Hybridization was conducted overnight in fresh solution also containing 100 μg/ml salmon sperm DNA (Sigma, St. Louis, MO) at 42°C with [α-^{32}P]-labeled probe. Hybridized membranes were washed twice in 1$\times$ SCC at room temperature for 5 min, twice in 1$\times$ SCC/0.1% SDS at 65°C for 30 min, and once in 0.1$\times$ SCC at room temperature for 15 min. Autoradiography (XAR Kodak film, Sigma) was performed at -70°C.

Rhinovirus inactivation

UV light inactivation of virus was accomplished by exposure to 1200 μJ/cm^2 UV light for 30 min. Confirmation of inactivation was conducted by microtiter plate assays using the methods described above.

Inactivation of the virus was also investigated by determination of new viral protein synthesis by [^{35}S]methionine incorporation and immunoprecipitation as follows. Live virus and UV-inactivated virus were inoculated onto 10^7 Ohio HeLa cells at an MOI of 1. After 30-min adsorption, 10 ml of 10% MEM was added, and cells were incubated in 5% CO_2 at 37°C. After 6 h of incubation, the medium was replaced by methionine-free medium (Sigma). Thirty minutes later, [^{35}S]methionine (DuPont-New England Nuclear) was added to a final concentration of 50 μCi/ml. Incubation was continued for 2 h, and the culture was then placed on ice. After being washed three times, cell pellets were collected and suspended in 1 ml of lysis buffer (1% Triton X-100 in PBS). Lysates were stored in 250-μl aliquots at -70°C for immunoprecipitation. Immunoprecipitation was performed on a 250-μl lysate aliquot, which was thawed, and an additional 750 μl of lysis buffer was added. Lysates were then sonicated and incubated at 4°C for 10 min. To reduce nonspecific binding, 50 μl of Staph protein A/Sepharose Fast Flow (Sigma) was added, and lysates were agitated at 4°C for 1 h, protein A/Sepharose was pelleted at 12,000 rpm in a microfuge for 1 min, and supernatant was removed. The protein A/Sepharose/polyclonal Ab complex (prepared by adding 5 μl of guinea pig polyclonal anti-rhinovirus type 9 Ab (ATCC) to 100 μl of Staph protein A-Sepharose and agitating at room temperature for 2 h) was then added to the supernatant, and agitation was continued at 4°C overnight. After overnight incubation, pellets were washed three times in PBS, and 50 μl of sample buffer (24% glycerol, 5% SDS, 12% β-ME, 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 Staph protein A/Sepharose pellet was removed. 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, then in EnHance (DuPont-New England Nuclear) for 1 h, and washed for 30 min in water. The gel was dried, and autoradiography was performed.

Rhinovirus inactivation was also performed by precoating the virus with soluble receptor to occupy all the receptor binding sites on the virus capsids. Virus stock solutions were preincubated with recombinant sICAM-1 (donated by P. Esmon, Bayer Corp, Berkeley, CA) at a concentration of 1 mg/ml for 30 min at room temperature. Confirmation of inactivation was conducted by microtiter plate assays using the methods described above.

Rhinovirus infectivity in A549 cells

Rhinovirus titrations. A549 cells were cultured at 2×10^6/ml in 2 ml of medium on 12-well plates, and when 80% confluent, cells were inoculated with rhinovirus at an MOI of 1. After 30 min of incubation at room temperature to allow virus attachment, cells were washed three times, and fresh medium was added. Supernatants were harvested at 6, 24, 48, and 72 h and stored at -70°C. Rhinovirus titers were determined using microtiter plate

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[4] Abbreviations used in this paper: $TCID_{50}$, tissue culture 50% infective dose; MOI, multiplicity of infection; CPE, cytopathic effect; sICAM, soluble intercellular adhesion molecule; NBF, neutral buffered formalin.

assays as described previously (25), including rhinovirus type 9 neutralizing antiserum (ATCC) to confirm the identity of the virus particles produced as rhinovirus type 9. Cultures were observed and stained with crystal violet to detect virus-induced CPE.

Rhinovirus genomic and replicative strand RNA analysis by RT-PCR.
A549 cells were cultured at 2×10^6/ml in 2 ml of medium on 12-well plates and, when 80% confluent, were inoculated with rhinovirus at an MOI of 1. After 30 min of incubation at room temperature to allow virus attachment, cells were washed three times, and fresh medium was added. Supernatants were then harvested immediately and at 6, 24, 48, 72, 96, and 120 h and stored at $-70°$C. Total RNA was extracted from 50-μl aliquots of supernatant using Tryzol (Life Technologies) according to the manufacturer's instructions. Rhinovirus genomic (positive) and replicative (negative) strand RNAs were detected using RT-PCR and internal probe hybridization as previously described (27). For genomic strand RT-PCR, the primer OL27 was used for the RT step, and PCR was conducted as previously described (27), except that the probe hybridization was conducted using nonisotopic labeling with the Amersham ECL kit (Amersham, Aylesbury, U.K.) according to the manufacturer's instructions. For the replicative strand RT-PCR, identical methods were used, except that the primer used for the RT step was OL26 (27).

Intracellular rhinovirus replication analysis by in situ hybridization.
A549 cells were cultured in 100-mm plates and, when 80% confluent, were inoculated with rhinovirus at an MOI of 1. After 30 min of incubation at room temperature to allow virus attachment, cells were washed three times, and fresh medium was added. Cells were then harvested by gentle scraping immediately and at 6 and 24 h. Cells were washed in PBS and fixed in neutral buffered formalin (NBF) for 1 h at 4°C. Cells were then washed three times in PBS and resuspended in 0.2-ml clots of human plasma (4). Cell clots were fixed in NBF for 24 h and embedded in paraffin wax blocks. Five-micron sections were hybridized with 200 ng/ml of a mixture of four conserved rhinovirus-specific probes (28). The probe sequences used were: PB4, CAG GGG CCG GAG GAC TCA AGA TGA GCA CAC GCG GCT; PB5, TGC AGG CAG CCA CGC AGG CTA GAA CTC CGT CGC CG; PB6, ACA CGG ACA CCC AAA GTA GTT GGT CCC ATC CCG CAA; and PB7, ACA TCC TTA ACT GGG TCT GTG AAT TTA CTG GGG TCT.

Rhinovirus new protein synthesis. Rhinovirus new viral protein synthesis in A549 cells was assessed by [^{35}S]methionine incorporation and immunoprecipitation as described above. Live virus was inoculated onto 10^7 A549 cells at an MOI of 1, and incubations with [^{35}S]methionine in methionine-free medium were performed for 2 and 24 h.

Viability of rhinovirus-infected A549 cells. A549 cells were cultured in 100-mm plates and when 80% confluent were inoculated with rhinovirus at an MOI of 1. After 30 min of incubation at room temperature to allow virus attachment, cells were washed three times, and fresh medium was added. The medium was then removed from infected and control noninfected cells at various time points. Detached cells in the supernatant were counted, attached cells on the plates were trypsinized and then counted, and trypan blue exclusion was determined to assess viability. For viability, 300 cells were counted after incubation with trypan blue for 5 min, and a ratio of living to dead cells was derived.

Statistical analysis

Data are expressed the mean $\pm$ SEM. Testing for significance in the time-course studies was performed using two-way repeated measures analysis of variance, followed by Student's paired t test at each time point. Other comparisons were conducted using Student's paired t test. $p \leq 0.05$ was considered significant.

Results

IL-8 release in response to rhinovirus

To determine whether IL-8 protein was released from A549 cells in response to rhinovirus in a dose-response manner, A549 pulmonary epithelial cells were cultured to 80% confluence in 12-well tissue culture plates and incubated with 10-fold dilutions of rhinovirus type 9 for 24 h. Supernatants were then harvested and assayed for IL-8. As shown in Figure 1*A*, the pulmonary epithelial cell line released IL-8 in a dose-dependent manner in response to rhinovirus. An MOI of 1 produced the greatest IL-8 release; studies were not conducted at higher MOIs to determine whether this was maximal. All additional experiments were performed at this dose of virus.
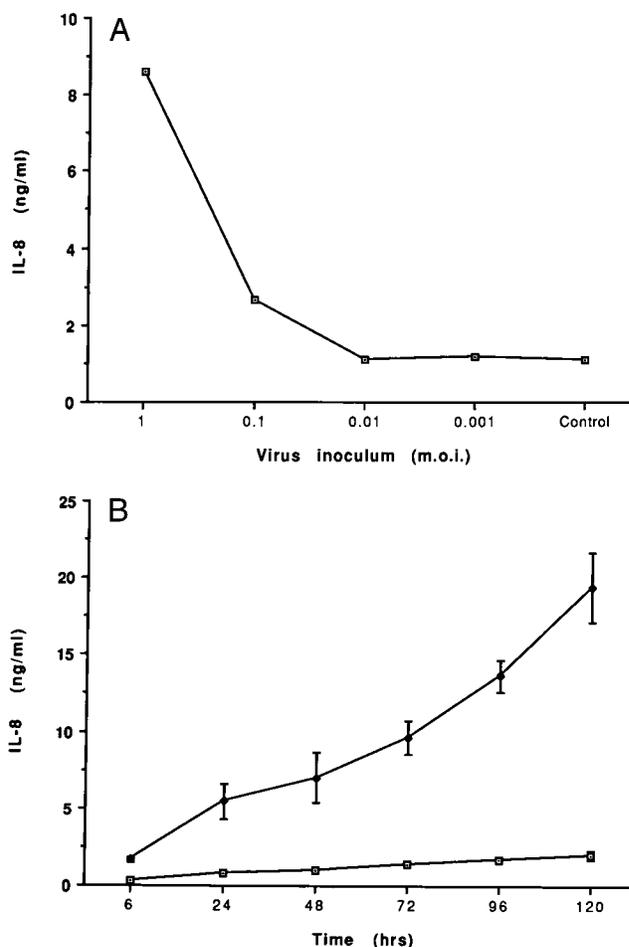
FIGURE 1. *A*, Dose-response of rhinovirus type 9-induced IL-8 protein release from A549 cells. IL-8 was measured by ELISA in supernatants from A549 epithelial cells cultured with medium alone (control) or with rhinovirus type 9 at doses ranging from 0.001 to 1 MOI. Supernatants were harvested for analysis at 24 h. *B*, Time course of rhinovirus type 9-induced IL-8 protein release from A549 cells. IL-8 was measured by ELISA in supernatants from A549 epithelial cells cultured with medium alone (open squares) or with rhinovirus type 9 at an MOI of 1 (closed squares). Supernatants were harvested for analysis at 6, 24, 48, 72, 96, and 120 h postinoculation. Data are the mean $\pm$ SEM of three separate experiments. Rhinovirus type 9 induced a significantly greater release of IL-8 compared with control release at all time points ($p < 0.001$).

We then investigated the time course of release of IL-8 from A549 cells in response to rhinovirus. A549 pulmonary epithelial cells were cultured to 80% confluence in 12-well tissue culture plates and incubated with rhinovirus type 9. Supernatants were then harvested at various time points and assayed for IL-8. There was a time-dependent release of IL-8 in response to rhinovirus. As shown in Figure 1*B*, IL-8 release was already elevated above the control value at 6 h and continued to increase until 120 h. Cultures were not continued beyond 120 h.

IL-8 mRNA induction in response to rhinovirus

To determine whether the observed IL-8 protein release induced by rhinovirus was accompanied by increased expression of IL-8 mRNA, we investigated IL-8 mRNA induction by rhinovirus using Northern analysis. A549 epithelial cells were cultured in 100-mm tissue culture plates. Rhinovirus type 9 was added, and RNA was harvested at 1, 3, 6, 24, 48, and 96 h. Northern blot analysis was performed to detect the presence of IL-8 mRNA. The time course
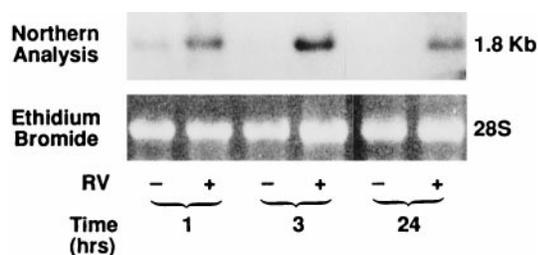
FIGURE 2. Time course of rhinovirus type 9-induced IL-8 mRNA expression in A549 cells. Representative Northern blot analysis of IL-8 mRNA (1.8 kb) in A549 epithelial cells incubated with rhinovirus type 9 (RV) at an MOI of 1. RNA was harvested for analysis at 1, 3, and 24 h. Rhinovirus induced increased expression of IL-8 mRNA in A549 cells, which was clearly visible at 1, 3, and 24 h after inoculation.

of IL-8 mRNA induction in response to rhinovirus type 9 up to 24 h is shown in Figure 2. A consistent response to rhinovirus type 9 was noted, with a detectable increase in mRNA at 1 h and a peak between 3 and 24 h. Induction of IL-8 mRNA expression in response to rhinovirus was still present at 48 h, although reduced compared to that at earlier time points. No induction was detected at 96 h (data not shown).

Inactivation of rhinovirus

As the rhinovirus replicative cycle is thought to be 6 to 8 h (29), we wished to investigate whether the observed induction of IL-8 protein was a response to virus replication or to virus-receptor binding triggering intracellular signaling pathways. To investigate this, we used two methods of inactivation of rhinovirus: UV inactivation to abolish rhinovirus replication and precoating the virus with solubilized receptor (sICAM-1) to prevent virus receptor binding (30).

Confirmation of UV and sICAM inactivation of rhinovirus type 9 was conducted in microtiter plate titration assays as described above. In the titration assays, the titer of stock rhinovirus type 9 was 2×10^6 TCID$_{50}$/ml, while that of inactivated virus by either method was zero, with no CPE observed even with undiluted inactivated virus stocks.

Inactivation of virus protein synthesis by UV light was also confirmed by immunoprecipitation of ^{35}S-labeled newly synthesized viral proteins produced in Ohio HeLa cells. Untreated rhinovirus type 9 demonstrated active new virus protein synthesis between 6 and 8 h after virus inoculation (Fig. 3, *lane 2*), while there was a complete absence of new viral protein synthesis with UV inactivated rhinovirus type 9 (Fig. 3, *lane 3*).

IL-8 release and mRNA induction in response to inactivated rhinovirus type 9

To investigate whether inhibition of virus replication by UV light altered rhinovirus induction of IL-8 protein release and mRNA expression, A549 epithelial cells were incubated with UV-inactivated and live rhinovirus type 9 and with medium alone. Supernatants were harvested at 24 and 120 h and were analyzed for IL-8 release by ELISA. RNA was harvested at 24 h and was analyzed by Northern analysis.

IL-8 protein release and mRNA induction are shown in Figures 4 and 5. UV-inactivated rhinovirus type 9 and control medium resulted in significantly less IL-8 protein release than live virus at both time points. However, UV-inactivated virus did result in increased IL-8 release compared with that using medium alone, with IL-8 levels being approximately half those of live rhinovirus type 9 (Fig. 4).
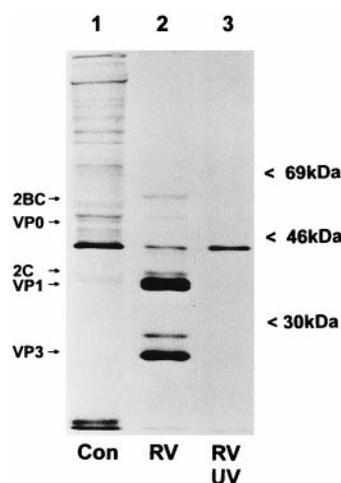
FIGURE 3. Effect of UV inactivation on rhinovirus type 9 replication (new protein synthesis) in Ohio HeLa cells. Newly synthesized, ^{35}S-labeled rhinovirus type 9 proteins were produced during a 2-h incubation with [^{35}S]methionine in methionine-free medium, conducted 6 h after inoculation of Ohio HeLa cells with control medium (*lane 1*), live rhinovirus type 9 (*lane 2*), or UV-inactivated rhinovirus type 9 (*lane 3*). Following immunoprecipitation with specific polyclonal antiserum, labeled proteins were run on an SDS-page gel. The gel was then dried and autoradiographed. Newly synthesized virus capsid proteins (principally VP1 and VP3, identified on the *left side*) are shown in *lane 2* (live rhinovirus type 9), but not in *lane 1* (medium alone) or in *lane 3* (UV-inactivated rhinovirus type 9).

The UV-inactivated virus also resulted in less IL-8 mRNA induction at 24 h than live virus, and medium alone induced less mRNA than either live or inactivated virus (Fig. 5). Again, the UV-inactivated virus produced approximately half the signal of the live virus.
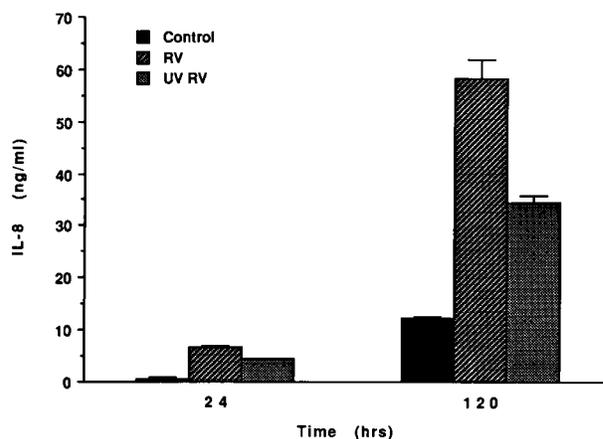
FIGURE 4. Effect of UV inactivation on rhinovirus type 9-induced IL-8 protein release from A549 cells. IL-8 was measured by ELISA in supernatants from A549 epithelial cells cultured with medium alone (control; filled bars), live rhinovirus type 9 at an MOI of 1 (RV; hatched bars), or UV-inactivated rhinovirus type 9 (UV RV; dotted bars). Supernatants were harvested for analysis at 24 and 120 h after inoculation. Data are the mean $\pm$ SEM of four experiments. Rhinovirus type 9 induced a significantly greater release than UV-inactivated virus or medium alone at both time points measured ($p < 0.01$), and UV-inactivated virus induced a significantly greater release than medium alone at both time points ($p < 0.01$).
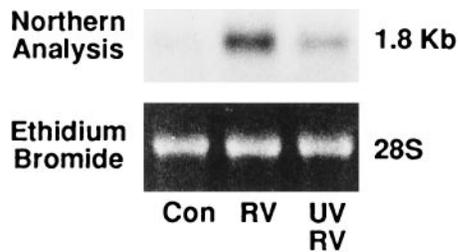
FIGURE 5. Effect of UV inactivation on rhinovirus type 9-induced IL-8 mRNA expression in A549 cells. Northern blot analysis of IL-8 mRNA (1.8 kb) in A549 epithelial cells cultured with medium alone (Con), live rhinovirus type 9 at an MOI of 1 (RV), or UV-inactivated rhinovirus type 9 (UV RV). RNA was harvested for analysis at 24 h. As observed previously (Fig. 3), rhinovirus induced increased expression of IL-8 mRNA in A549 cells that was clearly visible at 24 h after inoculation. This rhinovirus-induced up-regulation was reduced appreciably (~50%), but not completely, by UV inactivation of rhinovirus replication.

These results raised the possibility that some of the IL-8 release and mRNA production were occurring independently of viral replication, possibly as a result of virus-receptor interactions or of stimulation of the epithelial cells by factors other than virus in the inoculum. To investigate these possibilities, A549 cells were incubated with sICAM-inactivated and live rhinovirus type 9 and with medium alone. Supernatants were harvested at 24 and 120 h and were analyzed for IL-8 release by ELISA. There was no significant induction of IL-8 release by sICAM-inactivated rhinovirus compared with control medium (Fig. 6), while live rhinovirus resulted in significantly increased IL-8 release as previously observed.

Rhinovirus infection of A549 cells

The results reported above suggested that at least part of the induction of IL-8 protein and mRNA may result from rhinovirus replication in A549 cells. However, the ability of rhinoviruses to
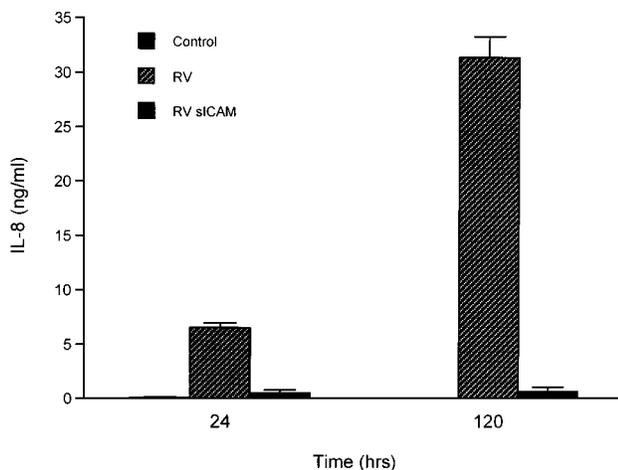
FIGURE 6. Effect of sICAM inactivation on rhinovirus type 9-induced IL-8 protein release from A549 cells. IL-8 was measured by ELISA in supernatants from A549 epithelial cells cultured with medium alone (control; filled bars), live rhinovirus type 9 at an MOI of 1 (RV; hatched bars), or sICAM-inactivated rhinovirus type 9 (sICAM RV; dotted bars). Supernatants were harvested for analysis at 24 and 120 h. Data are the mean ± SEM of three experiments. Rhinovirus type 9 induced a significantly greater release compared with control and sICAM-inactivated virus at both time points measured ($p < 0.01$). There was no significant difference between control and sICAM-inactivated virus at either time point ($p = $ NS).
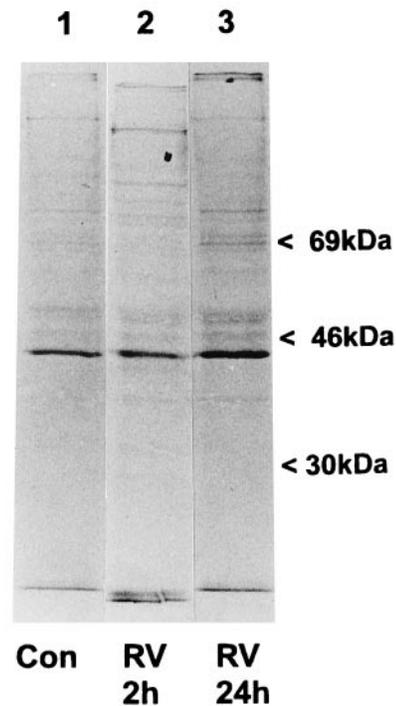
FIGURE 7. Immunoprecipitation of newly synthesized rhinovirus type 9 proteins from A549 epithelial cells. Newly synthesized, ^{35}S-labeled rhinovirus type 9 proteins were produced during a 2-h (*lane 2*) and a 24-h (*lane 3*) incubation with [^{35}S]methionine in methionine-free medium, conducted 6 h after inoculation of Ohio HeLa cells with control medium (*lane 1*) or live rhinovirus type 9 (*lanes 2* and *3*). Following immunoprecipitation with specific polyclonal antiserum, labeled proteins were run on an SDS-page gel. The gel was then dried and autoradiographed. In contrast to HeLa cells (Fig. 4, *lane 2*), no newly synthesized virus proteins are seen in either in *lane 2* (2-h incubation) or in *lane 3* (24-h incubation).

replicate in lower respiratory epithelium is controversial. To investigate the capacity of rhinovirus type 9 to replicate in the pulmonary epithelial cell line A549, we conducted studies to investigate new viral protein production, release of viral RNA into cell supernatants, intracellular localization of viral RNA, and release of functional viral replicative units into the supernatant.

Production of new rhinovirus proteins

The production of newly synthesized viral proteins following rhinovirus inoculation onto A549 cells was investigated by immunoprecipitation of ^{35}S-labeled newly synthesized viral proteins. Immunoprecipitation after incubation of rhinovirus type 9 with Ohio HeLa cells in the presence of ^{35}S-labeled methionine for 2 h (between 6–8 h after infection; Fig. 3, *lane 2*) produced strong bands for several newly synthesized viral proteins. Similar new protein synthesis was also observed with 24-h incubation with Ohio HeLa cells (between 6–30 h after infection) (31). In contrast, when rhinovirus type 9 was incubated with A549 cells under exactly the same conditions there was no detectable new viral protein synthesis after either the 2-h (Fig. 7, *lane 2*) or the 24-h (Fig. 7, *lane 3*) incubations. We concluded that immunoprecipitation was not sensitive enough to detect new viral proteins under the conditions described, and therefore we investigated viral protein production by Western analysis. Once again increased rhinovirus protein synthesis was easily detected when rhinovirus type 9 was inoculated onto Ohio HeLa cells for 24 h, but no increase in viral proteins was observed with rhinovirus type 9 inoculation onto A549 cells for the same time period (data not shown).
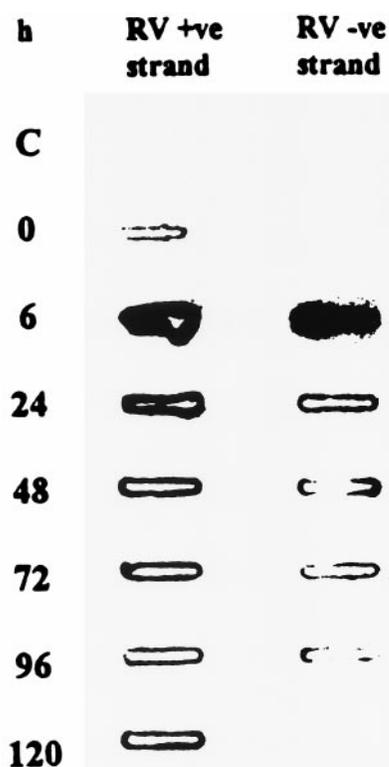
FIGURE 8. Detection of rhinovirus genomic (+ve) and replicative (−ve) strand RNAs by RT-PCR in supernatants from A549 pulmonary epithelial cells. Rhinovirus type 9 was inoculated at an MOI of 1 onto A549 cells and adsorbed for 30 min. Cells were washed three times, and fresh medium was added. Supernatants were harvested before inoculation (C), immediately after washing (time zero), and at 6, 24, 48, 72, 96, and 120 h after washing. RT-PCR and probe hybridization for genomic (+ve) and replicative (−ve) strands was conducted. A representative radiograph from four experiments is shown. A very weak signal for genomic (+ve) strand RNA can be seen immediately after washing (time zero), followed by a major increase in signal at 6 h. Thereafter, genomic RNA is still detectable up to and including 120 h after washing. For the replicative (−ve) strand, there is no signal immediately after washing, but again a strong increase in signal at 6 h, followed by a decline until signal, are undetectable 120 h after washing.

Production of rhinovirus RNA

As the protein detection techniques of immunoprecipitation and Western analysis were unable to detect rhinovirus replication in A549 cells, we elected to use more sensitive methods and investigated the release of rhinovirus RNA into the supernatants of A549 cells with RT-PCR assays for the genomic and replicative strands of rhinovirus RNA.

A549 cells were inoculated with rhinovirus; after attachment, cells were washed three times and fresh medium was added. Supernatants were harvested at various time points, and genomic and replicative strand RT-PCRs were conducted. There was no detectable replicative (−ve) strand RNA immediately following addition of fresh medium after washing (time zero, Fig. 8); there was then a marked increase in replicative strand RNA at 6 h after inoculation, followed by a gradual decline, until 96 h, after which no replicative strand RNA was detected. The results for the genomic (+ve) strand were similar; however, there was a weak signal present at time zero, presumably from the inoculated virus, again there was a marked increase in viral RNA at 6 h, followed by a gradual decline to 120 h, when only a weak signal was still detectable (Fig. 8).

Detection of intracellular rhinovirus replication by in situ hybridization

To ensure that the observed increases in viral RNA in the supernatants from A549 cells were not a result of either virus adsorption onto the cell surface followed by release of viral uncoated RNA after washing of the cells or of inefficient washing leading to persistence of viral RNA from the inoculum without active intracellular replication, we investigated intracellular rhinovirus replication using in situ hybridization.

A549 cells were cultured and inoculated with rhinovirus. Cells were then harvested at 0, 6, and 24 h; resuspended in plasma clots; fixed; and embedded in paraffin wax blocks. Sections were then examined by in situ hybridization for the genomic (+ve) strand of rhinovirus RNA. As shown in Figure 9, there was no detectable signal in control noninfected cells or in cells harvested immediately after inoculation and washing. At 6 and 24 h after inoculation, increases in intracellular rhinoviral RNA expression were clearly seen, demonstrating that new virus genomic RNA was being produced inside the cells.

Production of functional rhinovirus replicative units

Finally, we investigated the ability of A549 cells to assemble functional viral replicative units and to release them into the cell supernatant. Rhinovirus type 9 was inoculated onto A549 cells and adsorbed for 30 min. Following washing to remove unattached virus, fresh medium was added. Supernatants were harvested at 6, 24, 48, 72, 96, and 120 h. The production of functional viral replicative units was assessed by microtiter plate titration assays of cell supernatants as described above. Identification of the infectious units as rhinovirus type 9 was confirmed by neutralization with rhinovirus type 9-specific antiserum (ATCC). No infective virus was detectable in supernatants from cells at 6 h after inoculation. Peak titers were observed in A549 cell supernatants at 24 h after inoculation (Fig. 10); viral replication was just detectable in the supernatants at 48 h postviral inoculation, but not thereafter.

Viability of rhinovirus-infected A549 cells

To determine whether rhinovirus replication in A549 cells had any cytotoxic or cytopathologic effect, A549 cell cultures were infected with rhinovirus and observed for the development of CPE. A549 cell cultures were observed up to 120 h postviral inoculation, and at no stage was any rhinovirus-related CPE detected. Monolayers were also stained with crystal violet, and no disruption of the cell layer was observed at any time point.

In addition, the effects of viral infection on cell viability were examined by trypan blue exclusion. Rhinovirus type 9 was inoculated onto A549 cells and adsorbed for 30 min. Following washing to remove unattached virus, fresh medium was added. Supernatants and attached cells were harvested from infected and control noninfected cells at 24, 48, 72, 96, and 120 h. Detached cells in the supernatant were 100% trypan blue positive, and numbers were counted to assess shedding of nonviable cells by the monolayer. At no time point was there any difference between infected and noninfected supernatants (Table I). Attached cells on the plates were trypsinized and counted, and trypan blue exclusion was determined to assess viability. At no time point was there any difference in cell viability between the infected and noninfected attached cells (Table I).
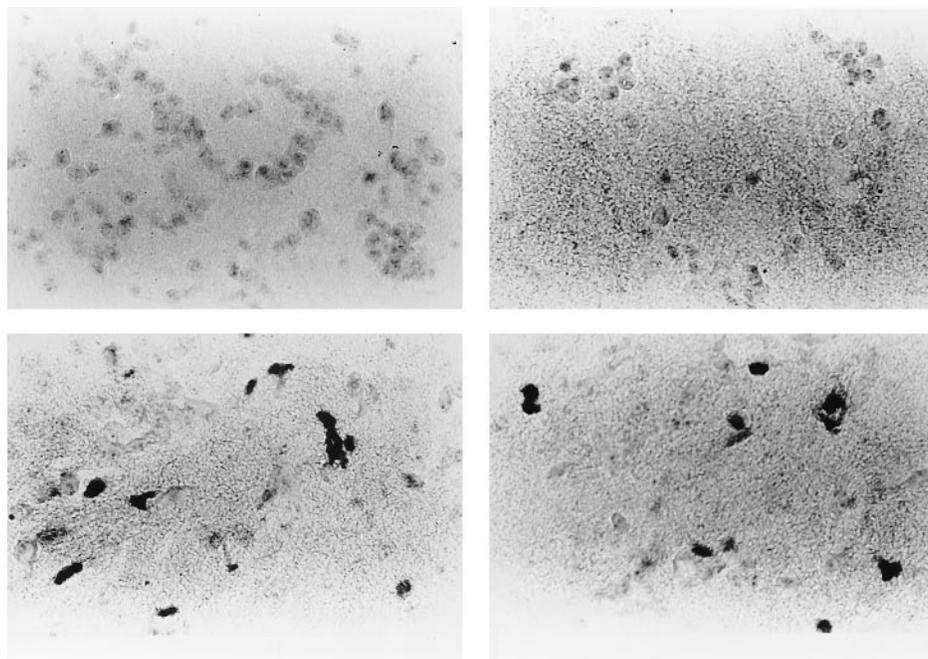
FIGURE 9. Detection of intracellular rhinovirus replication in A549 cells by in situ hybridization. Rhinovirus type 9 was inoculated at an MOI of 1 onto A549 cells and adsorbed for 30 min. Cells were washed three times, and fresh medium was added. Cells were harvested before inoculation (*upper left panel*) and immediately (*upper right panel*), at 6 h (*lower left panel*), and at 24 h (*lower right panel*) after washing. Cells were fixed, suspended in plasma, and paraffin embedded before in situ hybridization for genomic (+ve) strand RNA was conducted. A positive signal for rhinovirus RNA is indicated by a black coloration of the cells. No rhinovirus RNA is detectable in the cells of either control cells or cells immediately after inoculation and washing (*upper left* and *right panels*, respectively). Active intracellular rhinovirus replication is indicated by clear strong signals for rhinovirus RNA at both 6 h (*lower left panel*) and 24 h (*lower right panel*) after rhinovirus inoculation and washing. Magnification, ×400.

Discussion

These studies demonstrate that rhinovirus type 9 can infect the transformed pulmonary epithelial cell line A549, but that the infection is noncytopathic and low grade and appears to abort spontaneously 24 h after virus inoculation. They further show that rhi-

FIGURE 10. Production of rhinovirus type 9 infectious units from A549 pulmonary epithelial cells. Rhinovirus type 9 was inoculated at an MOI of 1 onto A549 cells and adsorbed for 30 min. Cells were washed three times, and fresh medium was added. Supernatants were harvested at 6, 24, 48, and 72 h, and virus titer was measured by microtiter plate assay. The cytopathic effect was confirmed to be rhinovirus type 9 with specific antiserum. Data are the mean $\pm$ SEM of three experiments. No virus infectious units were detectable at 6 h after inoculation and washing; however, by 24 h, there was clear production of replicative virus, with a peak titer of 64 $TCID_{50}$. By 48 h, replicative virus was almost undetectable, and by 72 h, no infectious units were detected.

novirus type 9 induces a prolonged time- and dose-dependent increase in IL-8 protein release from the pulmonary epithelial cell line. This release of IL-8 is associated with an induction of IL-8 mRNA. The inductions of both IL-8 protein release and mRNA expression were partially dependent on viral replication, being reduced by approximately 50% when virus replication was inhibited by UV inactivation. However, prevention of rhinovirus-receptor binding completely suppressed rhinovirus induction of IL-8 protein release.

There are several previous reports demonstrating that epithelial cell lines release IL-6, IL-8, IL-11, and GM-CSF following respiratory syncytial virus infection (32–35). Choi and Jacoby found increased IL-8 gene expression in human tracheal cells exposed to influenza virus (36). The role of rhinoviruses in promoting lower airway proinflammatory responses has received less attention until recent years, as rhinoviruses were until then thought to be principally limited to upper respiratory tract infections and the common cold. The recent data implicating rhinovirus infections in the majority of asthma exacerbations in both adults and children (1–3) has stimulated renewed interest in this virus type and its ability to infect the lower respiratory tract and provoke airway inflammation. In this regard, rhinovirus induction of cytokine protein and mRNA from the A549 pulmonary epithelial cell line has recently been demonstrated for IL-6 (10) and IL-11 (13); however, these studies did not examine the relationship between cytokine release and the ability of rhinoviruses to infect this cell line.

The majority of other respiratory viruses (adenovirus, influenza virus, respiratory syncytial virus, parainfluenza virus, and CMV) can infect the lower respiratory tract. However, evidence that this occurs with rhinoviruses is not conclusive. In vivo studies provide some support for this hypothesis, in that small particle aerosols of rhinovirus have been shown to produce tracheobronchitis (37).

Table I. *Effect of infection with rhinovirus type 9 on cell viability of A549 pulmonary epithelial cells*[a]

Time (h)	Cells in Supernatant (all nonviable cells) (number ($\times 10^5$/ml) mean $\pm$ SEM)[b]		p (control vs RV)	Attached Cells (percentage of nonviable cells by trypan blue exclusion) (percentage mean $\pm$ SEM)[b]		p (control vs RV)
	C	RV		C	RV	
24	2.0 ± 0.3	1.6 ± 0.2	NS	0.8 ± 0.5	1.0 ± 0.4	NS
48	2.0 ± 0.5	1.8 ± 0.2	NS	1.2 ± 0.1	1.3 ± 0.2	NS
72	4.0 ± 1.0	3.8 ± 0.7	NS	1.2 ± 0.3	1.1 ± 0.3	NS
96	4.8 ± 0.4	4.3 ± 1.3	NS	1.3 ± 0.4	0.8 ± 0.3	NS
120	7.3 ± 0.7	7.2 ± 0.6	NS	1.2 ± 0.1	1.3 ± 0.2	NS

[a] C, control; RV, rhinovirus type 9; NS, not significant.
[b] Mean $\pm$ SEM of three experiments.

Cultures of sputum from children with wheezy bronchitis were more often positive for rhinovirus than were nasal swabs taken at the same time (38). In experimental infection studies, rhinoviruses have been cultured from bronchial lavage fluid (39), and more recently rhinovirus has been demonstrated by RT-PCR in cells recovered from bronchial lavage (16). However, in both these studies the possibility of contamination from the upper airway during the nasal introduction of the bronchoscope could not be excluded. Finally, rhinoviruses are associated with a variety of severe lower respiratory tract diseases, such as bronchiolitis and pneumonia (40–42), and have been isolated from lower respiratory postmortem specimens in a few case reports (15).

There is also some in vitro evidence to suggest that rhinoviruses are capable of replicating in lower respiratory epithelium. Subauste et al. studied the transformed bronchial epithelial cell line BEAS-2B and used virus titrations in supernatants and lysates of cell pellets, and RT-PCR on RNA extracted from the cell pellets to demonstrate an increase in the rhinovirus genomic (+ve) strand for the first 24 h after virus inoculation and increases in virus titrations up to 72 h postinoculation (23). Interestingly, the infection of BEAS 2B cells was noncytolytic, and there was no effect on cell viability.

In this study, we have demonstrated that rhinovirus type 9 is also capable of initiating productive infection of the transformed pulmonary epithelial cell line, A549. This was demonstrated by the finding of the release of replicative (−ve) strand RNA into A549 cell supernatants, the demonstration of intracellular replication by in situ hybridization, and the release of functional viral replicative units into the supernatant. However, the infection was low grade, with virus being detectable only in neat supernatants in microtiter plate assays, and the maximal titer observed at 24 h postinoculation was 64 TCID$_{50}$/ml (Fig. 10). Similar to the BEAS 2B cells (23), no CPE on the A549 cells was observed at any time point; there was also no effect on cell viability (Table I). However, in contrast to the BEAS 2B cells, in which viral replication appeared to continue up to the final time point examined (72 h), infection of A549 cells was terminated spontaneously around 24 h. Despite this, IL-8 protein release was noted to increase up to 120 h after virus inoculation (Fig. 1*B*), while IL-8 mRNA expression tended to follow the pattern of infection more closely, peaking early between 3 and 24 h (Fig. 2) and returning to control levels by 96 h (data not shown). This prolonged release of IL-8 may be a result of changes in cellular function resulting in IL-8 protein release that persist for some time even after the infection itself has resolved.

The observations that both UV-inactivated and live rhinovirus are able to trigger IL-8 protein release and mRNA expression (although the induction with UV-inactivated virus was about 50% that with live virus; Figs. 4 and 5) suggest that rhinovirus binding to its cell surface receptor, ICAM-1, may be responsible in part for the increased IL-8 protein and mRNA. An alternative explanation would be that the virus stocks contained other active compounds, such as other cytokines. However, assays sensitive to <5 pg for likely candidates, including IL-1 and TNF, were negative (data not shown). Furthermore, the fact that induction of IL-8 protein release was completely suppressed by preventing virus-receptor binding by precoating the virus with sICAM suggests that the inoculum itself contained no other stimulatory substances that were able to induced IL-8 protein release or mRNA expression under these conditions. The possibility that cross-linking of ICAM-1 by rhinovirus occurs is made likely by the fact that each virus particle has 60 receptor binding sites (43). Further it has been demonstrated that cross-linking of ICAM-1 by Ab leads to both intracellular signaling (44) and cytokine release (45). We therefore believe that the virus-receptor interaction is the most likely explanation for the induction of IL-8 mRNA and protein release observed with UV-inactivated virus, while the difference in IL-8 protein release and mRNA induction observed between UV-inactivated and live virus is probably due to viral replication within the pulmonary epithelial cell line.

The observations of rhinovirus-induced IL-8 protein release and mRNA expression from a pulmonary epithelial cell line probably have relevance to human rhinovirus infections, as we have found increased levels of IL-8 in nasal aspirates during wild-type upper respiratory virus infections, the majority of which were due to rhinoviruses (14). We studied nasal secretions obtained during virus-induced exacerbations of asthma in school age children and found increased levels of neutrophil myeloperoxidase and IL-8 in the presence of rhinoviral infection. Interestingly, there were correlations between the levels of IL-8 and neutrophil myeloperoxidase and between myeloperoxidase and the severity of upper respiratory symptoms, suggesting that IL-8 may play an important role in neutrophil chemoattraction and activation in rhinovirus infections (14). These observations are supported by an experimental rhinovirus infection study in asthmatic subjects, in whom increased levels of nasal IL-8 were observed after rhinovirus infection (12). This study was also of particular interest, as the researchers found correlations between the levels of nasal IL-8 and the increases in bronchial hyper-reactivity induced by rhinovirus infection, again suggesting that IL-8 may play an important role in the pathogenesis of rhinovirus-induced asthma exacerbations. The findings of the present study and those reported by Subauste et al. (23) suggest that rhinovirus-induced release of IL-8 from lower airway epithelium may be an important source of this chemokine.

Eosinphil infiltration is an important feature of asthma (18), and there is increasing evidence that eosinophil recruitment and activation are also important in virus-induced asthma exacerbations. We have found increased levels of eosinophil major basic protein in nasal aspirates taken from children with rhinovirus-induced asthma exacerbations (L. M. Teran, M. C. Seminario, G. J. Gleich, and S. L. Johnston, unpublished observations). Calhoun et al. demonstrated increased allergen-induced bronchial lavage eosinophil numbers in atopic rhinitic subjects, compared with those in normal subjects, in the presence of rhinovirus experimental infections (19). We also investigated the lower airway cellular response to rhinovirus infection using experimentally induced rhinovirus infections in normal and asthmatic subjects. Both normal and asthmatic subjects had increased numbers of eosinophils in the bronchial mucosa at 4 days after rhinovirus infection. However, in the asthmatic subjects, the eosinophil infiltrate was still present when the subjects were rebiopsied 6 to 8 wk later, while in normal subjects, eosinophil numbers had returned to baseline levels (17). Given the possible role of IL-8 in eosinophil recruitment and activation (21, 22), and the fact that its role in this respect may be up-regulated in asthma (46), it is possible that this chemokine plays an important role in the recruitment and activation of both eosinophils and neutrophils in the context of rhinovirus-induced asthma exacerbations.

A prominent feature of both normal and asthmatic subjects in the experimental rhinovirus infection study was the increased numbers of $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes in the bronchial mucosa at 4 days after rhinovirus infection (17). IL-8 is also a lymphocyte chemoattractant (47); it is therefore possible that rhinovirus-induced IL-8 secretion from pulmonary epithelium plays a role in the recruitment and activation of several cell types involved in the pathogenesis of virus-induced asthma. However, further definition of the role of this chemokine in virus-induced asthma exacerbations will come from clinical studies making assessments of the inter-relationships between IL-8 levels and the severity of clinical illness and of viral infection in normal and asthmatic subjects (48).

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