Rhinovirus stimulation of interleukin-8 in vivo and in vitro: role of NF-κB

ZHOU ZHU, WEILIANG TANG, JACK M. GWALTNEY, JR., YANG WU, AND JACK A. ELIAS
Section of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06520-8057; and Division of Epidemiology and Virology, Department of Internal Medicine, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

Zhu, Zhou, Weiliang Tang, Jack M. Gwaltney, Jr., Yang Wu, and Jack A. Elias. Rhinovirus stimulation of interleukin-8 in vivo and in vitro: role of NF-κB. *Am. J. Physiol.* 273 (*Lung Cell. Mol. Physiol.* 17): L814–L824, 1997.—Neutrophil infiltration is a well-documented early event in the pathogenesis of rhinovirus (RV) infections. To further understand the mechanisms responsible for this neutrophilia, we determined whether interleukin (IL)-8 was present at sites of experimental RV infection in vivo and characterized the mechanism(s) by which RV stimulates IL-8 production in vitro. IL-8 was readily detectable in the nasal washings of all normal volunteers and did not increase with sham nasal inoculation. In contrast, RV infection caused a significant additional increase in nasal IL-8, the levels of which peaked 48–72 h after virus inoculation. RV was a potent stimulator of IL-8 protein production by A549 epithelial-like cells, MRC-5 fibroblasts, and normal human bronchial epithelial cells in vitro. This induction was associated with a significant increase in IL-8 mRNA accumulation and gene transcription. RV also stimulated IL-8 promoter-driven luciferase activity. This stimulation was significantly decreased by mutation of the nuclear factor (NF)-IL-6 site and was completely abrogated by mutation of the NF-κB site in this promoter. In addition, NF-κB-DNA binding activity was rapidly induced in RV-infected cells. This inducible binding was made up of p65 and, to a lesser extent, p50 NF-κB moieties. These studies demonstrate that IL-8 is present in normal nasal secretions and that the levels of IL-8 are further increased after RV infection. They also demonstrate that RVs are potent stimulators of IL-8 production and that this induction is mediated, at least in part, by an NF-κB-dependent transcriptional activation pathway. IL-8 may contribute to the pathogenesis of RV infection, and NF-κB activation may be a central event in RV-induced pathologies.

nuclear factor-κB; nose; epithelial cell; airway

RHINOVIRUS (RV) infections are the most common acute infectious illness in humans (5, 16, 38). They account for 5–20% of the acute illnesses in the developed world, including 50–60% of the common colds (22, 43) and 40% of the flairs of chronic bronchitis (38). They are also important precipitants of otitis media, sinusitis, and asthma (5, 11, 21, 22, 29). Studies using polymerase chain reaction (PCR)-based diagnostics have recently emphasized the importance of viruses in the latter by demonstrating that viruses are responsible for ~80–85% and 45% of the asthma flairs in 9- to 11-yr-old children and adults, respectively, with RV being the most commonly implicated pathogen (21, 29). Thus RV infections are a major cause of lost work days, school absenteeism, and health care expenditure.

Animal models of RV infection are not readily available (5, 38). As a result, the pathogenesis of RV infection has been investigated largely in naturally occurring and experimental RV infections in otherwise normal volunteers. These studies have demonstrated that the nasal mucosa is the primary site of RV infection (5, 38). They have also demonstrated that neutrophil infiltration is an important early event in RV infection because increased numbers of neutrophils can be detected in the mucosa and nasal secretions of patients with RV upper respiratory tract infections (28, 43, 44). In contrast to a variety of other respiratory pathogens (influenza, adenovirus, etc.), cell cytotoxicity does not play a major role in the pathogenesis of RV infections (5, 7, 17, 38, 44). Instead, it is believed that the manifestations of RV-induced pathologies are the result of virus-induced mediators of inflammation. Support for this hypothesis comes from studies from our laboratories and others demonstrating an increase in kinins, interleukin (IL)-1, and IL-6 in nasal washings from patients with RV infection (28, 33, 45). The soluble mediators responsible for the recruitment of neutrophils during the course of RV infections are, however, poorly defined.

IL-8 is a pleiotropic chemokine that is produced by a large variety of cells in response to a wide array of stimulants (reviewed in Refs. 6 and 23). Among its most notable features are its neutrophil chemotactic and activating properties. In keeping with these properties, IL-8 plays an important role in the generation of acute neutrophilic infiltrates, and dysregulated IL-8 production has been implicated in the pathogenesis of a variety of pulmonary disorders, including the adult respiratory distress syndrome, idiopathic pulmonary fibrosis, asthma, and cystic fibrosis (reviewed in Ref. 6). IL-8 has been documented in vivo in the nasal washes of children with respiratory syncytial virus (RSV) infections (31). In addition, a variety of viruses, including RV, have been shown to stimulate epithelial cell IL-8 elaboration in vitro (2, 21, 40). However, the ability of RV to stimulate IL-8 production in vivo and the mechanism by which RV stimulates IL-8 elaboration remain poorly understood.

To further understand the cellular events involved in the pathogenesis of RV respiratory tract infections, studies were undertaken to determine whether RV induced IL-8 production in vivo and in vitro, and the mechanism of the induction was characterized. These studies demonstrate that IL-8 can be detected in the nasal lavages of normal volunteers and that the levels of IL-8 increase after experimental RV infection. They

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also demonstrate that RVs are potent inducers of bronchial and lung epithelial cell IL-8 elaboration and nuclear factor (NF)-κB activation and that RV-induced IL-8 production is mediated largely via an NF-κB-dependent transcriptional activation process.

MATERIALS AND METHODS

RV Challenge of Volunteers

The clinical study protocol that was employed has been previously described (33, 45). In brief, healthy young adults 18 yr of age or older with reciprocal serum neutralizing antibody titers less than or equal to two to the challenge virus were recruited from the University of Virginia student body. Viral challenges were performed by administering 0.25-ml volumes of either RV type 39 or RV strain Hanks (not neutralized by antisera to 89 numbered RV types, acid sensitive, and chloroform-idoxuridine resistant) two times in each nostril. A total dose of 800 tissue culture infectious dose 50 ($TCID_{50}$) of RV strain Hanks or 2,500 $TCID_{50}$ of RV type 39 was employed. Starting on the morning before challenge and at 24-h intervals thereafter, all volunteers were interviewed with regard to the presence and severity of the following 10 symptoms: sneezing, nasal discharge, nasal congestion, malaise, headaches, chills, feverishness, sore throat, hoarseness, and cough. Symptoms were rated for severity on a scale from zero to three. Patients were designated as having a "cold" if they had a total symptom score of equal or greater than five over the 5 days after challenge plus either nasal discharge for 3 days or the belief of the subject that a cold had occurred (20). Sham inoculation was carried out in the same manner except that Hanks' balanced salt solution instead of viral solution was used.

Nasal lavages were performed with 10 ml of isotonic saline one time per day on study *day 0* (before challenge) to *day 5*. One portion of the lavage fluid was used for viral culture. The other aliquot was stored at −70°C until its IL-8 content was assayed. Serum samples were also obtained from some selected individuals at 24-h intervals on *days 0-5*. They were also stored at −70°C until assayed for IL-8. Viral culture was accomplished by combining lavage fluid with concentrated veal infusion broth and inoculating monolayers of MRC-5 human fetal lung fibroblasts (BioWhittaker, Walkersville, MD). One isolate from each subject was identified as the challenge virus using a standard neutralization test.

Homotypic neutralizing antibody titers were determined by standard tests on blood collected before and 3 wk after inoculation (18). Volunteers were considered infected if they shed virus or had a fourfold or greater rise in serum antibody titer. All of the RV-inoculated volunteers in these studies became infected. This allowed us to compare volunteers that became infected and had colds (infected/ill volunteers) and volunteers that were infected but did not manifest colds (infected/non-ill volunteers).

Viral Stock Preparation

RV14 was obtained from the American Type Culture Collection (ATCC; Rockville, MD). Viral stocks were prepared by infection of sensitive cell systems with a low-input multiplicity of infection (MOI). When the infection was advanced, cell supernatants were harvested, cells were disrupted by freezing and thawing, and debris was pelleted by low-speed centrifugation. Aliquots of clarified supernatants were frozen at −70°C. Viral adsorption was performed at 37°C. Incubations were performed at 33°C for RV. Titers of infectivity of stock viruses were determined by inoculation of serial dilutions into sensitive cell systems and quantification of plaque formation as previously described (19). No detectable levels of IL-1β, tumor necrosis factor-α, and endotoxin were found in the stocks.

For selected experiments, RV stock preparations were further purified using sucrose gradients. To accomplish this, virus stock was concentrated by centrifugation at 150,000 *g* at 4°C for 45 min using a Beckman L5–50 centrifuge and a SW50.1 rotor (Beckman Instruments, Palo Alto, CA). The resulting viral pellet was resuspended in NTE buffer [in mM: 150 NaCl, 50 tris(hydroxymethyl)aminomethane (Tris)·HCl (pH 7.4), and 1 EDTA] and was overlaid onto a two-layer sucrose cushion containing 2.4 ml of 60% sucrose in NTE in the bottom layer and 2.4 ml of 30% sucrose in NTE in the top layer. Centrifugation was then repeated for 90 min, the interface containing virus was collected and filter sterilized, viral titers were quantified via plaque assay, and purified virus was separated into aliquots and stored at −70°C until utilization.

Cell Infection and Supernatant Preparation

A549 alveolar epithelial type II-like cells and MRC-5 human fetal lung fibroblasts were obtained from ATCC and were grown to confluence in 100-mm petri dishes in Dulbecco's modified Eagle's medium supplemented with nonessential amino acids, L-glutamine, penicillin, streptomycin (DMEM), and 10% fetal bovine serum (FBS; Hyclone Laboratory, Logan, UT). Normal human bronchial epithelial cells (NHBE) and serum-free bronchial epithelial cell growth medium (BEGM) with supplements were purchased from Clonetics (San Diego, CA). NHBE cells from different donors were cultured according to the supplier's instructions, and only cells of *passages 2* and *3* were used for the experiments. On the day of infection, the cell culture medium was aspirated, and cultures were inoculated with virus stock at an MOI of 0.1–3.0. After adsorption at 37°C for 90 min, the viral solution was removed, the cells were rinsed with phosphate-buffered saline (PBS), DMEM with 2% FBS or BEGM (for the NHBE cells) was introduced, and the cells were incubated at 33°C for the desired period of time. The supernatants were removed at designated time points, clarified by low-speed centrifugation, and stored at −70°C until analysis. The cell monolayers were then rinsed with PBS and were harvested for mRNA analysis as described below.

IL-8 Quantification

Immunoreactive IL-8 was quantitated using a dual antibody enzyme-linked immunosorbent assay (ELISA) kit obtained from R & D Systems (Minneapolis, MN) according to the manufacturer's protocol. The sensitivity limit of these kits is 10 pg/ml.

Assessment of Cell Viability

Cell viability was assessed by trypan blue dye exclusion.

RNA Isolation and Analysis

Total cellular RNA was extracted from cell monolayers at desired time points using the acid-guanidinium thiocyanate-phenol-chloroform extraction protocol described by Chomczynski and Sacchi (4). Equal amounts (20 µg) of RNA from each experimental condition were size fractionated by electrophoresis through 1% agarose and 17% formaldehyde gels, transferred to nylon membranes, and hybridized with [^{32}P]cDNA probes. Clone 19.1, containing a full-length IL-8 cDNA, was a gift from Dr. T. Yoshimura, National Cancer Institute, Frederick, MD. The 240-bp *Pst* I/*Eco*R I fragment of the coding

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region of this clone was excised and was used as a probe (26). It was labeled to a high specific activity [10^9 counts·min^{-1} (cpm)·μg DNA^{-1}] using the random primer method, and its binding was assessed after a wash under conditions of increasing stringency [low stringency: 2× standard sodium chloride and sodium citrate (3 M NaCl, 0.3 M Na_3 citrate·$2H_2O$) (SSC), 0.1% sodium dodecyl sulfate (SDS), 42°C, 15 min, two times; medium stringency: 1× SSC, 0.1% SDS, 42°C, 15 min, two times; and high stringency: 0.1× SSC, 0.1% SDS, 42°C, 15 min, two times] and autoradiography. The adequacy of gel loading was routinely assessed by ethidium bromide staining.

Nuclear Run-On Analysis

The relative rates of nuclear transcription were assessed using modifications of procedures previously described (9, 45). Confluent cell monolayers (25 × 10^6 cells/sample) were incubated in the presence and absence of RV at 33°C for the noted period of time. The cells were then washed two times with PBS, mechanically detached, pelleted, and resuspended in lysis buffer [in mM: 10 Tris·HCl (pH 7.4), 2 $MgCl_2$, 3 $CaCl_2$, 3 dithiothreitol (DTT), and 300 sucrose, as well as 0.5% Triton X-100]. The nuclei were then harvested by centrifugation, resuspended in 100 μl of storage buffer [50 mM Tris·HCl (pH 8.3), 5 mM $MgCl_2$, 0.1 mM EDTA, and 40% glycerol], and stored at −70°C until further analyzed. Nylon membranes carrying 20 μg each of isolated cDNA fragments encoding IL-8, granulocyte macrophage colony-stimulating factor and pUC-18 without a cDNA insert, and 3 μg of cDNA encoding 28S were prepared using a MINIFOLD II slot-blotting apparatus and were baked in a vacuum oven at 80°C for 2 h. In vitro transcription and RNA labeling were carried out in transcription buffer [20 mM Tris·HCl (pH 8.3), 100 mM KCl, 4.5 mM $MgCl_2$, 2 mM DTT, and 400 μM each of ATP, GTP, and CTP] in the presence of 200 μCi [^{32}P]UTP at 30°C for 30 min. The reaction was stopped by adding 1 μl of 100 mM UTP and by chasing at 30°C for 10 min. Total RNA in the reaction solution was isolated using guanidinium isothiocyanate and phenol-chloroform as described above. Dried RNA pellets were dissolved in 100 μl of TE buffer (10 mM Tris·HCl and 1 mM EDTA, pH 7.8), and radioactivity was determined using 1 μl of the solution. Hybridization was performed by the incubation of equal numbers of counts of radiolabeled RNA with DNA immobilized on nylon membranes as described above. The membranes were washed at high stringency, and binding was evaluated using autoradiography.

Reporter Gene Constructs

The IL-8 promoter-luciferase constructs used in these studies were modified from IL-8 promoter-chloramphenicol acetyltransferase (CAT) constructs provided by Dr. Charles Kunsch (Human Genomic Sciences, Rockville, MD). The original constructs pIL-8(WT)CAT, pIL-8(−κB)CAT, and pIL-8(−NF)CAT contained the wild-type, NF-κB mutant, and NF-IL-6 mutant sequences of the IL-8 promoter cloned into the *Xho* I and *Bam*H I sites of the pBLCATPA vector (24). The promoter fragments between nucleotides −420 and +101 in each of these constructs were amplified by PCR using the IL-8 promoter-CAT constructs as templates and a set of primers (5′-ATT GCG TGG ATC CTC TTC ACC ATC ATG ATA GC-3′; 5′-ATA TCT CGA GTT TAC ACA CAG TGA GAA TGG TTC CT-3′) designed to introduce *Bam*H I/*Xho* I restriction sites in the 5′- and 3′-ends, respectively. The PCR products that were obtained were then digested, gel purified, and ligated into the vector pXP2-luciferase (ATCC) using T4 ligase. All of the constructs were verified by dideoxy sequencing using an automated DNA sequencer. The nucleotides from −95 to −53 are as follows for each of the constructs used in our study: pIL-8(WT)LUC, TCA GTT GCA AAT CGT GGA ATT TCC TCT GAC ATA ATG AAA AGA T; pIL-8(−NF-κB)LUC, TCA GTT GCA AAT CCT GCA ATG TCG TCT GTC ATA ATG AAA AGA T; and pIL-8(−NF-IL-6)LUC, TCA GCT ACG CAG CGT GGA ATT TCC TCT GAC ATA ATG AAA AGA T.

Cell Transfection and Luciferase Assay

Plasmid DNA was introduced into A549 cells using a modification of the DEAE-dextran transfection protocol of Fashena et al. (13) as previously described. In this procedure, 1 × 10^6 A549 cells were seeded 16–24 h before transfection in 60-mm petri dishes in complete DMEM with high glucose and 10% FBS. The cells were then washed and were gently agitated for 30 min at room temperature in the presence of 4.5 μg DNA and 0.5 mg/ml DEAE-dextran in a volume of 300 μl. At the end of this incubation period, the cells were washed and were allowed to recover in DMEM for 24 h. Virus or control solution was then added, and the cells were incubated for 4 h at 33°C in 5% CO_2 and air. The cells were then washed with PBS, mechanically detached, pelleted, and resuspended in 100 μl of 0.25 M Tris·HCl (pH 7.8) in the presence of 25 μl of Lysis Reagent (Promega, Madison, WI). The lysates were then clarified by centrifugation and were stored at −20°C. Luciferase activity was measured using the luciferase assay system from Promega. Quantification was obtained in a Lumat model LB9501 luminometer (Bethold, Germany). Transfection efficiency was routinely accounted for by cotransfecting (1.5 μg) pCMV-β-gal (Clontech, Palo Alto, CA), a construct that contains the β-galactosidase gene driven by the cytomegalovirus immediate early promoter. β-Galactosidase activity was assessed using the chromogenic technique of Eustice et al. (10). All luciferase measurements were normalized for transfection efficiency using the β-galactosidase values. The resulting data are expressed as relative light units.

Electrophoretic Mobility Shift Assays

Preparation of nuclear extracts. Nuclear extracts were prepared using modifications of the techniques of Schreiber et al. (35). Uninfected and RV-infected A549 cells were prepared as noted above. At the desired points in time, the cells were mechanically detached, suspended in Tris-buffered saline [5 mM Tris·HCl (pH 7.8) and 0.15 M NaCl], freshly supplemented with protease inhibitors (1 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), pelleted at 4°C, and resuspended and swelled in *solution A* [in mM: 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 10 KCl, 0.1 EDTA, 0.1 ethylene glycol-bis(β-aminoethyl ether)-*N,N,N*′,*N*′-tetraacetic acid (EGTA), and 1 DTT with freshly added protease inhibitors as above] for 15 min on ice. Membrane lysis was accomplished by adding 25 μl of 10% Nonidet P-40 followed by vigorous agitation. The nuclei were then collected by centrifugation, resuspended in 50 μl of *solution B* [in mM: 20 HEPES (pH 7.9), 400 NaCl, 1 EDTA, 1 EGTA, and 1 DTT, with freshly added protease inhibitors as above], and agitated vigorously at 4°C for 15 min. After centrifugation, the supernatants were collected. The protein concentrations of the nuclear extracts were determined using the Bradford method, and the extracts were separated into aliquots and stored at −70°C until utilization.

Oligonucleotide probes. Four different NF-κB oligonucleotides were synthesized in the Oligo Synthesis Lab of Yale University for use in the electrophoretic mobility shift assays (EMSA). They included the recognition site for concensus NF-κB (5′-TCG ACA GAG GGG ACT TTC CGA GAG GC-3′), a mutant NF-κB (5′-TCG ACA GAG AAT ACT TTC CGA GAG

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GC-3′), the IL-8-promoter NF-κB sequence (5′-TCG TGG AAT TTC CTC TG-3′), and a mutated IL-8 promoter NF-κB sequence (5′-TCG TCG AAT TTC CTC TG-3′). Oligonucleotide probe for activator protein 1 (AP-1) (5′-CGC TTG ATG ACT CAG CCG GAA-3′) was purchased from Stratagene (La Jolla, CA).

Electrophoresis. EMSAs were performed using the techniques of Schreiber et al. (35). Radiolabeled double-stranded oligonucleotide probes were prepared by annealing complementary oligonucleotides and end labeling using [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolab, Beverly, MA). The labeled probes were purified by push-column chromatography, diluted with buffer [10 mM Tris·HCl (pH 8.0) and 1 mM EDTA] to the desired concentration, and incubated with equal aliquots of nuclear extract (2–5 µg) and 2 µg of poly[dI-dC]-poly[dI-dC] at room temperature for 30 min. Resolution was accomplished by the electrophoresis of 10 µl of the reaction solution (~10,000 cpm) on vertical 5 or 8% nondenaturing polyacrylamide gels containing 2% glycerol using 25% (vol/vol) TBE buffer [in mM: 22.3 Tris·HCl, 22.3 boric acid, and 0.25 EDTA, pH 8.0]. The gels were then dried, and binding activity was assessed via autoradiography.

Supershift EMSA. Supershift assays were used to determine which members of the NF-κB family were involved in RV-induced NF-κB-DNA binding. In these studies, EMSA were performed as described above except that rabbit polyclonal antibodies against the NF-κB subunit proteins p65, p50, p52, c-Rel, and Rel B (Santa Cruz Biotechnology, Santa Cruz, CA) were included in the 1-h radiolabeled probe-extract binding reaction (1 h, 4°C). Preimmune serum (Santa Cruz Biotechnology) was routinely employed to control for any nonspecific effects of these antisera.

RESULTS

IL-8 in Sham and RV-Inoculated Volunteers

To address the role of IL-8 in RV infection, nasal washings were obtained from normal volunteers before and at 24-h intervals after sham or RV nasal inoculation. The IL-8 protein levels in these washings were then evaluated by ELISA. In contrast to prior studies from our laboratory demonstrating that significant levels of IL-6 cannot be detected in nasal lavages of normal volunteers (45), IL-8 was readily detected in the nasal lavage fluid of all volunteers before sham or virus inoculation. In the 24 volunteers studied, the IL-8 levels ranged between 36 and 1,642 pg/ml. Overall, a mean ± SE of 283.6 ± 92.4 pg/ml of IL-8 was appreciated. The manipulation associated with nasal inoculation and lavage did not significantly alter these levels (Fig. 1). In contrast, RV infection caused a significant increase in nasal IL-8 levels. This increase was detected at 24 h and peaked 48–72 h after RV infection (Fig. 1).

The IL-8 that was present in the nasal secretions from RV-challenged patients could be locally produced. Alternatively, it could enter the nose from the circulation due to local permeability alterations induced by RV. To differentiate between these possibilities, we simultaneously collected nasal secretions and sera from RV-challenged volunteers and compared their IL-8 content. As can be seen in Table 1, IL-8 was readily detected in the nasal samples but not the serum

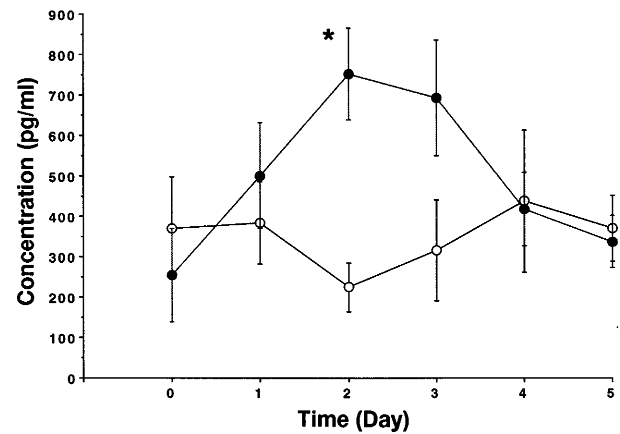

Fig. 1. Immunoreactive interleukin (IL)-8 in nasal washings from sham- and rhinovirus (RV)-challenged normal volunteers. Levels of IL-8 in the nasal washings of volunteers were quantitated before RV challenge (*day 0*) and at 24-h intervals thereafter. Values represent means ± SE of 6 sham-challenged (○) and 18 virus-challenged (●) individuals. *$P < 0.01$, Student's *t*-test comparing sham- and RV-challenged groups.

samples. This suggests that the IL-8 detected in the nasal secretions is locally produced.

IL-8 in Volunteers With and Without Colds

The pathophysiology of the symptoms experienced by RV-infected individuals is poorly understood. To address the role(s) that IL-8 might play in the generation of these symptoms, we compared the levels of IL-8 before and after RV infection in infected/ill and infected/non-ill volunteers. To our surprise, the baseline (prechallenge) levels of nasal IL-8 were significantly lower in patients that manifest a cold after RV infection (infected/ill) than in patients that did not meet cold criterion (infected/non-ill; Fig. 2). In contrast, the levels of IL-8 after infection were similar in both groups (Fig. 2).

RV Stimulation of IL-8 Protein Production and mRNA Accumulation In Vitro

To further investigate the relationship between RV infection and IL-8, studies were undertaken to determine if RV stimulates IL-8 production in vitro. Under normal culture conditions, uninfected A549 cells, MRC-5 cells, and NHBE cells produced small to modest amounts of IL-8 that accumulated slowly with time in culture. RV infection caused a rapid and impressive increase in the IL-8 production of each of these cells.

Table 1. *IL-8 in nasal and serum samples*

Patient No.	Sample	Days After RV Challenge 0	1	2	3	4	5
1	Nasal	175	360	4,024	3,161	2,203	1,055
	Serum	14	31	16	17	17	18
2	Nasal	383	6,557	5,929	6,040	2,936	5,384
	Serum	16	18	14	17	15	15

Values for interleukin-8 (IL-8) are picograms per milliliter and represent means of triplicates that were within 10% of each other. Nasal and serum samples were obtained from volunteers before and at 24-h intervals after rhinovirus (RV) challenge.

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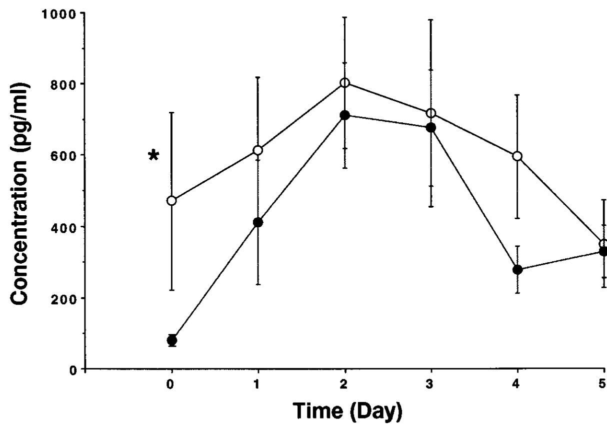

Fig. 2. Immunoreactive IL-8 in nasal washings from RV-infected volunteers with and without colds. Nasal washings were obtained from volunteers on the day before RV challenge (*day 0*) and at 24-h intervals thereafter. Levels of immunoreactive IL-8 in the fluids were determined by enzyme-linked immunosorbent assay. ●, Patients who developed clinically significant colds (infected/ill); ○, patients who did not manifest a cold (infected/non-ill). Values represent means ± SE of evaluations of 10 infected/ill and 8 infected/non-ill individuals. * $P < 0.05$, Student's *t*-test.

This induction could be appreciated as early as 4 h after viral inoculation, and the levels of IL-8 continued to increase with continued cell incubation (Fig. 3). The induction appeared to be, in great extent, virus mediated because purified RV14 also stimulated IL-8 production, and ultraviolet light exposure markedly diminished the inductive capacity of RV14 (data not shown).

Studies were also undertaken to determine if the conditions that induced IL-8 production altered cell viability. At baseline, A549 cells and NHBE cells were >97% viable as assessed by trypan blue dye exclusion. Interestingly, RV infection did not significantly alter A549 cell viability and did not induce visually detectable cytopathic alterations (data not shown). In contrast, RV caused significant cytopathic changes in NHBE cell cultures, and NHBE cell viability fell to 65% at the end of the 96-h incubation period.

Uninfected A549 cells contained levels of IL-8 mRNA that were at the lower limits of detection with the assays employed. In accordance with our findings with IL-8 protein, RV infection caused a rapid and significant increase in IL-8 mRNA accumulation. This induction was time-dependent and peaked 2 h after viral inoculation (Fig. 4). Similar accumulation of IL-8 mRNA was observed in NHBE cells (data not shown). These results clearly demonstrate that RV induction of IL-8 protein production in vitro is associated with a significant increase in IL-8 mRNA accumulation and is, in great extent, pretranslationally mediated.

RV Stimulation of IL-8 Gene Transcription

To further characterize the mechanism by which RV stimulates IL-8 production, nuclear run-on assays were performed to characterize the transcriptional effects of the virus. As can be seen in Fig. 5, a low level of IL-8 gene transcription appeared in nuclei from uninfected A549 cells, and this transcriptional activity increased

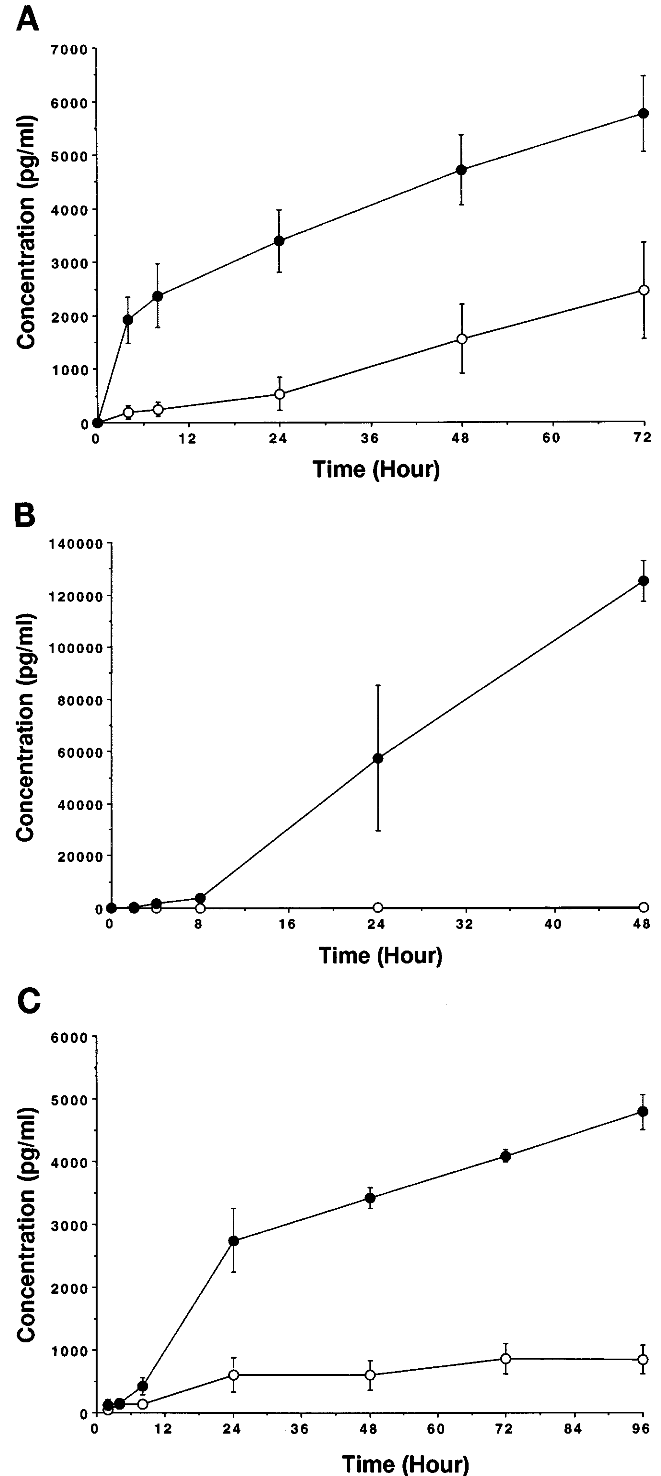

Fig. 3. Kinetics of IL-8 elaboration by RV-infected A549, MRC-5, and normal human bronchial epithelial cells (NHBE) cells. Monolayers of A549 lung epithelial-like cells (*A*), MRC-5 lung fibroblasts (*B*), and NHBE cells (*C*) were incubated in medium alone (○) or inoculated with RV14 [multiplicity of infection (MOI) = 1; ●]. Levels of IL-8 in the cell supernatants were quantitated at intervals thereafter. Values represent means ± SE of 4 independent experiments for A549 cells, 3 for MRC-5 cells, and 3 for NHBE cells. Data from uninfected and RV-infected cells for each time point were processed using Student's *t*-test ($P < 0.01$ for all time points after 24 h).

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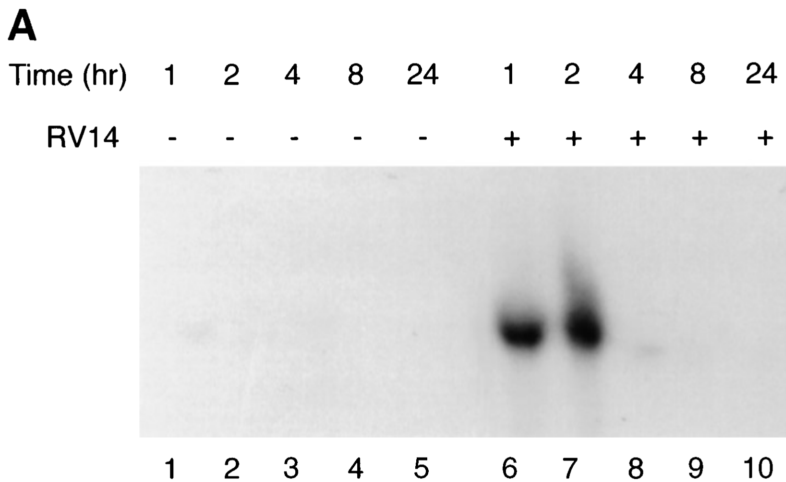

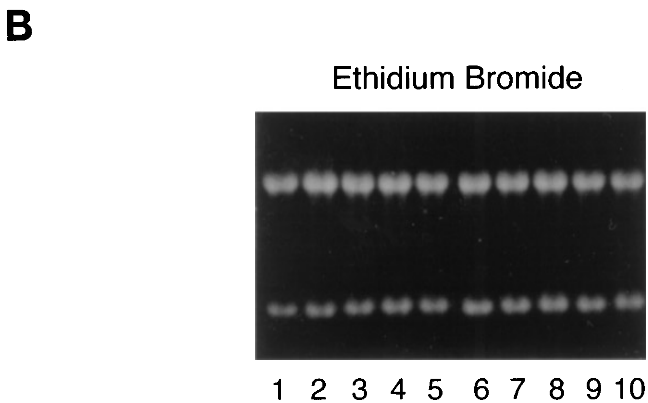

Fig. 4. Representative experiment (n = 3) demonstrating RV stimulation of IL-8 mRNA in A549 cells. A549 cells were uninfected or infected with RV14 at an MOI of 1. After adsorption, the cells were rinsed and incubated in complete medium with 2% fetal bovine serum. Levels of IL-8 mRNA in these cells at various time points after infection were evaluated as described in MATERIALS AND METHODS. *A*: levels of IL-8 mRNA in RV-infected (+) and medium control (−) cells. *B*: ethidium bromide loading controls.

significantly with RV infection. This demonstrates that RV stimulation of epithelial cell IL-8 production is, at least partially, transcriptionally mediated.

RV Stimulation of IL-8 Promoter Activity

Because transcriptional mechanisms play an important role in RV stimulation of IL-8 production, studies were initiated to define the *cis* element(s) in the IL-8 promoter responsible for these effects. IL-8 promoter-luciferase reporter gene constructs were prepared and were transfected into A549 cell monolayers. The luciferase activity in uninfected and RV-infected cells was then assessed. As seen in Fig. 6, a low level of luciferase

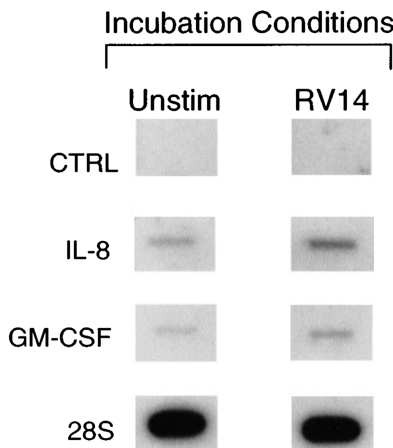

Fig. 5. Effect of RV14 on IL-8 gene transcription. Nuclei were obtained from uninfected (Unstim) and RV14-infected A549 cells 24 h after viral inoculation, and the relative rates of IL-8, granulocyte macrophage colony-stimulating factor (GM-CSF), and 28S gene transcription were ascertained as described in MATERIALS AND METHODS. Figure is representative of n = 3 experiments. Nonspecific hybridization was assessed by quantitating the binding to pUC-18 without a cDNA insert (CTRL, control).

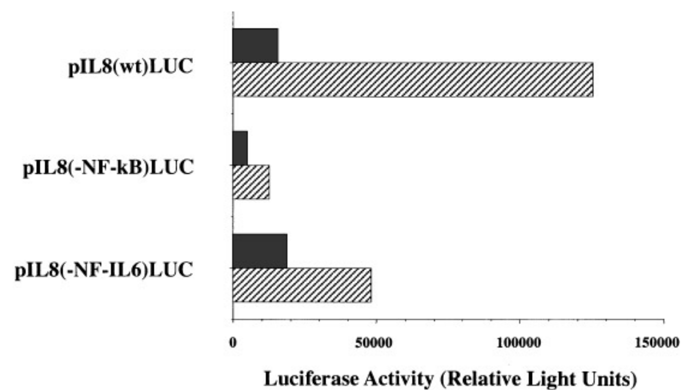

Fig. 6. IL-8 promoter-driven luciferase activity in A549 cells. A549 cells were transfected with IL-8 promoter-luciferase constructs, allowed to recover for 24 h, incubated in the presence or absence of RV14 (MOI = 1), and cultured for an additional 4 h. Cell layers were then harvested, and luciferase activity was assessed as described in MATERIALS AND METHODS. β-Galactosidase activity was also assessed, and these values were used to correct for transfection efficiency. Luciferase activity in uninfected cells (solid bars) and RV14-infected cells (hatched bars) containing constructs pIL8(wt)LUC, pIL8(−NF-κB)LUC, and pIL8(−NF-IL-6)LUC are illustrated. Figure is representative of n = 3 experiments.

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activity was detected in uninfected cells. In contrast, construct pIL8(WT)LUC, which contains the sequences in the IL-8 promoter between nucleotides −420 and +101, responded briskly to RV14. Mutation of the NF-IL-6 site [pIL8(−NF-IL-6)LUC] in this promoter region caused an ~50–60% decrease in luciferase activity. Furthermore, mutation of the NF-κB site [pIL8(−NF-κB)LUC] totally abrogated the response of this promoter construct to RV14. These results clearly demonstrate that RV response elements are present in the region between −420 and +101 in the IL-8 promoter. They also demonstrate that, in this region, the NF-IL-6 site plays a modest role and the NF-κB site plays a crucial role in RV induction of IL-8 promoter activity.

RV Induction of NF-κB

To further understand the NF-κB-dependent mechanism(s) by which RV stimulates IL-8 promoter activity, studies were undertaken to determine if RV could alter the NF-κB binding activity in nuclei from human lung epithelial cells. Nuclei from uninfected and infected A549 cells were isolated, lysates were prepared, and EMSA were performed using labeled wild-type and mutated IL-8-promoter NF-κB and consensus NF-κB sequences. Nonspecific binding was constitutively present in nuclear lysates from uninfected cells (Figs. 7 and 8). RV infection did not significantly alter this binding. It did, however, induce additional NF-κB binding activities that migrated as two new bands on 5% polyacrylamide gels (Figs. 7 and 8). These bands appeared to be Rel/NF-κB specific because unlabeled IL-8-promoter NF-κB sequences and consensus NF-κB sequences effectively competed, whereas mutated IL-8-promoter NF-κB sequences and AP-1 consensus sequences did not compete for DNA binding (Fig. 7). To identify the subunits that were activated in response to RV infection, EMSA were performed using antibodies against specific members of the NF-κB transcription factor family. These supershift assays demonstrated that antibodies to p65 completely supershifted both RV-induced NF-κB bands (Fig. 8), whereas antibodies to p50 shifted the smaller RV-induced DNA binding moiety. In contrast, antibodies to p52, c-Rel, Rel B, and preimmune serum did not alter RV-induced NF-κB binding (Fig. 8). These studies demonstrate that RV selectively induces NF-κB binding in lung epithelial cells and that p65 is a major and p50 is a lesser contributor to these NF-κB complexes.

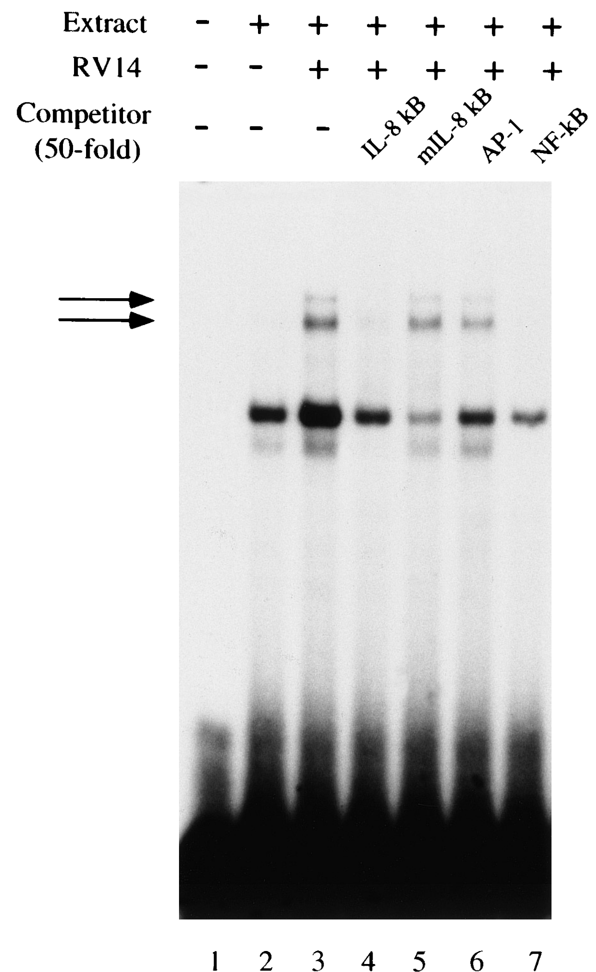

Fig. 7. Specificity of RV14-induced nuclear factor (NF)-κB binding activity. Nuclear lysates were prepared from uninfected and RV14-infected A549 cells. NF-κB binding activity in these lysates was assessed using radiolabeled IL-8 NF-κB sequences in the presence and absence of excess unlabeled IL-8 NF-κB motifs (IL-8 κB), mutated IL-8 NF-κB motifs (mIL-8 κB), consensus NF-κB motifs (NF-κB), and activator protein 1 motifs (AP-1). Virus-induced NF-κB DNA binding activities are highlighted by the solid arrows. Resolution was accomplished as described in MATERIALS AND METHODS.

DISCUSSION

RV infections are extremely common since, on average, adults experience 2–4 and children experience 4–10 infectious episodes per year (15, 27). Studies of naturally occurring and experimental infections have demonstrated that neutrophil infiltration is a reproducible early feature of RV infection (43, 44). They have also demonstrated that cell cytotoxicity is not a major contributor to RV pathologies (7, 17, 43, 44). This has led to the belief that the clinical and pathological features of RV infection are, in great extent, due to the elaboration by the host of a variety of inflammatory mediators. To further understand the processes responsible for the nasal neutrophilia seen during the course of RV infections, studies were undertaken to determine if the potent neutrophil chemotactic agent IL-8 was present in nasal secretions before and after RV infection. In vitro approaches were also used to determine if RV stimulates airway and respiratory epithelial cell and fibroblast IL-8 elaboration and to characterize the mechanism of the stimulation that was noted. These studies demonstrate that IL-8 is present in the nasal secretions of normal volunteers. They also demonstrate that the levels of nasal IL-8 increase after RV infection, but not after sham nasal inoculation and mechanical manipulation. Last, they demonstrate that RVs are potent stimulators of epithelial cell IL-8 production and NF-κB activation, that RV-induced IL-8 production is mediated, in great extent, by an NF-κB-dependent

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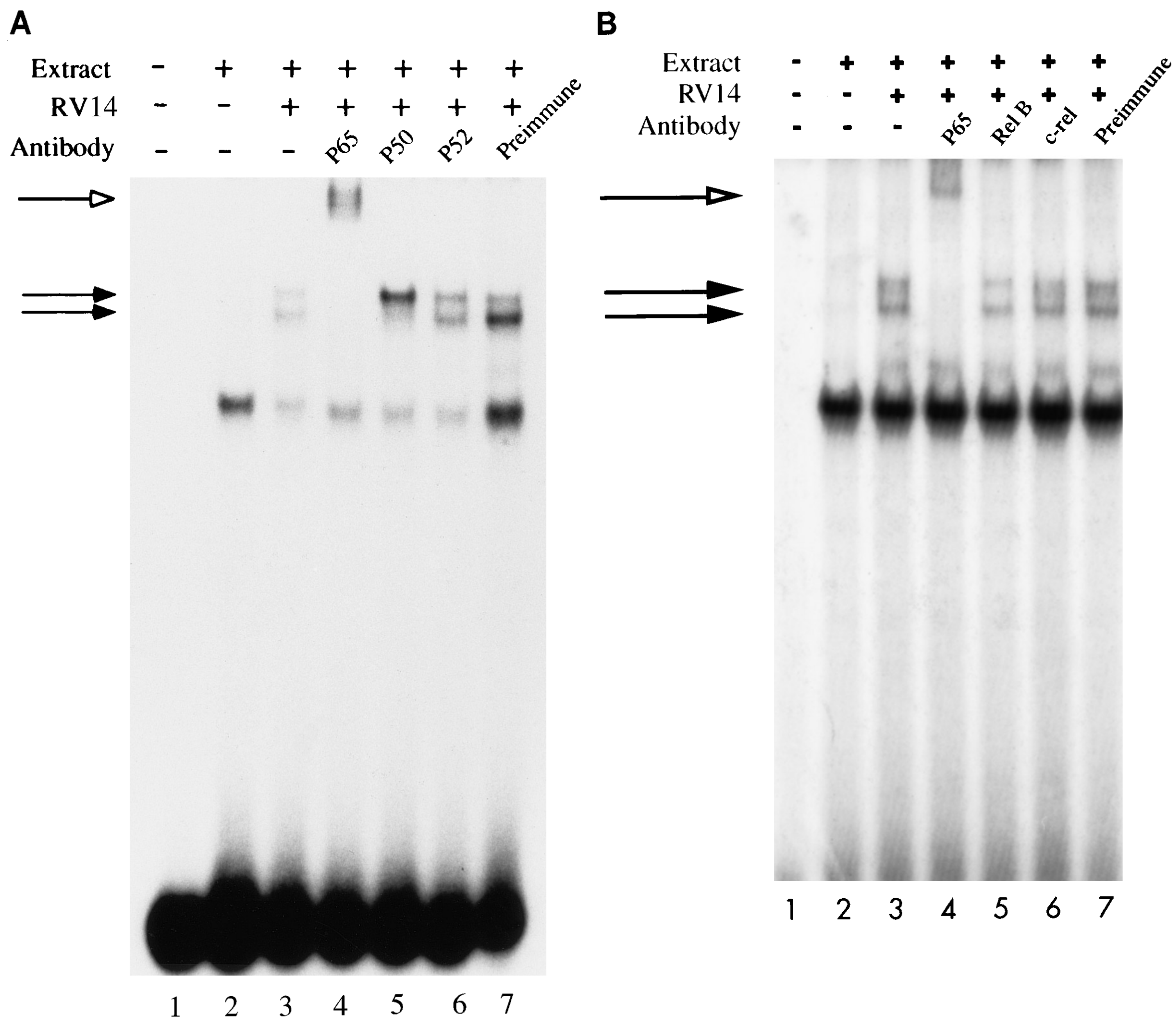

Fig. 8. Identification of RV-induced NF-κB moieties using supershift electorphoretic mobility shift assay (EMSA). Nuclear lysates were prepared from uninfected and RV14-infected A549 cells and EMSAs were performed in the presence and absence of antisera against NF-κB family proteins. Radiolabeled IL-8 NF-κB oligonucleotides were used in the reaction mixture. *A*: antisera against p65, p50, and p52 are compared with preimmune serum. *B*: antisera against p65, Rel B, and c-Rel are compared with preimmune serum. RV14-induced NF-κB binding activities are highlighted by the solid arrows. Supershifted moieties caused by antiserum against p65 are highlighted by the open arrows.

transcriptional activation process, and that the p65 and, to a lesser extent, p50 NF-κB moieties contribute to this activation. These studies are among the first to adequately define the IL-8 response at sites of RV infection. They are also the first to define in detail the transcriptional mechanism by which RV induces IL-8 elaboration.

To gain insight into the potential roles of IL-8 in RV infection, we compared the levels of IL-8 in the nasal washings from sham-inoculated volunteers and virus-infected volunteers that did (infected/ill) and did not (infected/non-ill) meet the criterion for colds. These studies demonstrate that, in contrast to sham inoculation and manipulation, RV infection caused a significant increase in nasal IL-8 levels. Interestingly, significant differences between the levels of nasal IL-8 in the infected/ill versus the infected/non-ill volunteers were not appreciated. These observations are in keeping with prior studies of experimental RV infections in which statistically significant differences in nasal neutrophil counts were appreciated when comparisons were made between infected/ill and sham inoculated volunteers but not when comparisons were made between infected/ill and infected/non-ill volunteers (28). All in all, these findings suggest that IL-8 may be a major contributor to RV-induced nasal neutrophilia. In addition, they suggest that IL-8 is not directly responsible for the generation of cold symptomatology in RV-infected patients.

RV infections are associated with multifaceted pathological lesions. In addition to nasal neutrophilia, subepithelial edema, serum protein exudation, and the enhanced accumulation of epithelial and submucosal lymphocytes and eosinophils are well documented (5, 14, 38, 42). In addition, RV infection augments the histamine release and eosinophil infiltration seen after endobronchial antigen challenge in sensitive patients (3). The pathogenesis of each of these abnormalities is

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poorly understood. It is interesting to note, however, that IL-8, in addition to its effects on neutrophils, has effects on a variety of other relevant cells. In particular, IL-8 is chemotactic for some T lymphocytes and basophils, induces tissue permeability alterations, and can stimulate the migration of eosinophils from patients with atopic dermatitis (6, 23). Thus IL-8 may also contribute to the generation of the lymphocytic and eosinophilic abnormalities seen during RV infections.

IL-8 is, in many ways, the prototypic neutrophil chemotactic and activating C-X-C subfamily chemokine (6, 23). It is not usually expressed in significant quantities in the normal lower respiratory tract (41) but is commonly expressed in the setting of neutrophilic inflammation (6, 23). Our studies, however, demonstrate that IL-8 is produced constitutively in the normal nose. The explanation for this in vivo activation is not clear. It is reasonable to believe, however, that it represents subclinical epithelial cell activation. This is in keeping with the known filtering function of the nose, which traps potentially activating microbes and particulates. It is also in accord with prior studies in children (30) and studies demonstrating that normal nasal epithelial cells constitutively express IL-8 mRNA (2) and that normal nasal secretions contain large numbers of neutrophils (12).

An unexpected finding in our studies was the observation that, before RV infection, patients that subsequently manifest cold symptomatology (infected/ill) had lower baseline levels of IL-8 than patients that did not manifest cold symptomatology (infected/non-ill). The biological significance of this observation is not clear. It is tempting to speculate, however, that elevated baseline levels of IL-8 are, in some way, protective and act to minimize the generation of RV-induced symptomatology. Documentation of the validity of this speculation and characterization of the mechanism(s) of this protection will require further investigation.

To further define the mechanism(s) by which RV stimulates cytokine production, in vitro approaches were used to define the events involved in RV stimulation of IL-8 elaboration. In accordance with other reports (21, 29), our studies demonstrated that RVs are potent stimulators of bronchial and alveolar epithelial cell IL-8 protein production. Our studies also demonstrated that RV-induced alterations in IL-8 protein production are associated with significant increases in IL-8 mRNA accumulation and gene transcription. Luciferase-reporter gene assays were then employed to define the *cis* elements in the IL-8 promoter region responsible for this induction. These studies demonstrated that RV is a potent stimulator of IL-8 promoter-luciferase activity and that this stimulation is totally abrogated by mutation of the NF-κB site in the promoter construct. Furthermore, EMSA and supershift EMSA demonstrated that RV induces NF-κB-DNA binding that is mediated, at least in part, by complexes containing p65 and p50, but not p52, c-Rel, or Rel B subunits. These observations demonstrate that RV stimulates IL-8 production via a transcriptional mechanism and that this process is largely NF-κB dependent. They also suggest that p65, and to a lesser extent p50, subunits of NF-κB play an important role in this stimulation.

It is important to note that in almost no case is a single NF-κB site sufficient to regulate target gene transcription. Activation typically depends on multiple transcription factors interacting in a combinatorial fashion (8, 36). Interactions between NF-κB and NF-IL-6 sites are well documented in transcriptional regulation (24, 25, 34). A direct physical association between these transcription factors has also been established (39). Our studies demonstrated that mutation of the NF-IL-6 site also caused a significant decrease in RV-induced IL-8 promoter activity. This suggests that the NF-IL-6 site also plays a role in RV induction of IL-8 elaboration. It also raises the possibility that maximal RV-induced IL-8 elaboration depends on NF-κB-NF-IL-6 interaction. An interaction of this sort has been shown to be important in the regulation of IL-8 production by phorbol-stimulated Jurkat cells (24).

Survival requires dynamic responses to environmental challenges and infectious agents. To mount these responses, an organism requires a sensitive and rapidly acting system to activate potentially lifesaving immune and inflammatory events. The Rel/NF-κB transcription factor family stands out as a central coordinator of these responses to stress and injury (36). Under most circumstances, these factors lay dormant in the cytoplasm, kept there by inhibitory (I)-κB proteins. They dissociate from I-κB in response to a wide variety of activating stimuli. This activation is a complex process that involves I-κB phosphorylation and in most cases ubiquitination, proteolytic degradation, and the translocation of NF-κB into the nucleus. The NF-κB subunits then form *trans* activating dimers among themselves and interact with other transcription factors to regulate the transcription of genes involved in inflammation, immunity, and stress responses (1, 32, 36, 37). Previous studies from our laboratory demonstrated that RV is a potent activator of NF-κB-DNA binding and that RV induction of epithelial cell IL-6 production was mediated via an NF-κB-dependent activation pathway (45). The present study confirms the ability of RV to activate NF-κB in lung epithelial cells and demonstrates the importance of NF-κB activation in IL-8 elaboration. When viewed in combination, these studies suggest that NF-κB activation is a central component in the pathogenesis of RV infections.

In summary, these studies demonstrate that IL-8 is produced constitutively in normal nasal secretions and that the levels of IL-8 are further increased after experimental RV infection. These studies also demonstrate that RV is a potent stimulator of bronchial and lung epithelial cell IL-8 elaboration and NF-κB activation and demonstrate that RV-induced stimulation of IL-8 production is the result of an NF-κB-dependent transcriptional activation process. These studies suggest that IL-8 plays a role in RV infection and that NF-κB activation may be an important event in RV-induced pathologies.

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Address for reprint requests: J. A. Elias, Section of Pulmonary and Critical Care Medicine, Dept. of Internal Medicine, Yale Univ. School of Medicine, 333 Cedar St., LCI 105, New Haven, CT 06520-8057.

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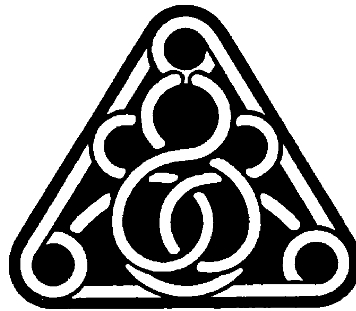

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