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Research Article

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Rhinovirus Stimulation of Interleukin-6 In Vivo and In Vitro

Evidence for Nuclear Factor κB-dependent Transcriptional Activation

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Abstract

To further understand the biology of rhinovirus (RV), we determined whether IL-6 was produced during RV infections and characterized the mechanism by which RV stimulates lung cell IL-6 production. In contrast to normals and minimally symptomatic volunteers, IL-6 was detected in the nasal washings from patients who developed colds after RV challenge. RV14 and RV1A, major and minor receptor group RVs, respectively, were potent stimulators of IL-6 protein production in vitro. These effects were associated with significant increases in IL-6 mRNA accumulation and gene transcription. RV was also a potent stimulator of IL-6 promoter-driven luciferase activity. This stimulation was modestly decreased by mutation of the nuclear factor (NF)–IL-6 site and abrogated by mutation of the NF-κB site in this promoter. An NF-κB–DNA binding activity, mediated by p65, p50, and p52 NF-κB moieties, was rapidly induced in RV-infected cells. Activator protein 1–DNA binding was not similarly altered. These studies demonstrate that IL-6 is produced during symptomatic RV infections, that RVs are potent stimulators of IL-6 elaboration, and that RV stimulation of IL-6 production is mediated by an NF-κB–dependent transcriptional stimulation pathway. IL-6 may play an important role in the pathogenesis of RV infection, and NF-κB activation is likely to be an important event in RV-induced pathologies. (*J. Clin. Invest.* 1996. 97:421–430.)
Key words: epithelial cell • fibroblast • NF-IL-6 • upper respiratory tract infection

Introduction

Rhinoviruses (RVs)[1] are the major cause of the common cold, the most common acute infectious illness in humans (1, 2). They also cause 40% of exacerbations of chronic bronchitis (1), have been epidemiologically linked to asthmatic exacerbations, and predispose to bacterial sinusitis and otitis (3). Thus, they are responsible for a significant portion of working days lost in industry, commerce, and education and significant morbidity and health care expenditure.

RV infections are species specific, and animal models of RV infection are not readily available. As a result, the pathogenesis of RV infection has been largely investigated in experimentally induced colds in normal volunteers. These studies have demonstrated that the nasal mucosa is the primary site of RV infection and suggest that RV-induced IFN-α and type-specific Ig, including IgA, contribute to viral recovery and clearance, respectively (4, 5). They have also shown that, in contrast to a variety of other respiratory pathogens (influenza, adenovirus, etc.), cell cytotoxicity does not appear to play a major role in the pathogenesis of RV infections since mucosal biopsies during RV infections reveal sparse inflammatory cell infiltration and few other histologic abnormalities (1, 2, 6–8). As a result, it is now believed that chemical mediators of inflammation play a major role in the pathogenesis of RV infection. Support for this hypothesis comes from studies from our laboratories and others demonstrating an increase in kinins and IL-1 in nasal washings from patients with RV infections (9, 10). Little else is known, however, about the mediators that are involved in the host response to RV infection. Importantly, virtually nothing is known about the mechanism(s) by which RV triggers the release of the inflammatory mediators that play such an important role in the pathogenesis of RV infections.

IL-6 is a pleiotropic cytokine that is produced by a wide array of cells in response to a large number of stimuli (11, 12). It plays an important role in the host response to stress and injury via its ability, among other things, to stimulate the acute phase response, to activate T lymphocytes, to augment the terminal differentiation and Ig production of B lymphocytes, and to function as an endogenous pyrogen. Regulated IL-6 production is an important component of normal biologic homeostasis, and dysregulated IL-6 production has been implicated in the pathogenesis of a wide variety of inflammatory, neoplastic, and viral disorders (11–13). Studies from our laboratory and others have demonstrated that IL-6 is a potent regulator of pulmonary inflammation and plays a critical role in the stimulation of mucosal IgA immune responses (14–17). These observations clearly demonstrate that IL-6 mediates many biological phenomena that are relevant to RV infection. The ability of RV to stimulate IL-6 production, however, has not been adequately investigated.

To understand further the cellular events involved in the pathogenesis of RV respiratory tract infection, studies were

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1. *Abbreviations used in this paper:* AP, activator protein; EMSA, electrophoresis mobility shift assay; HSV, herpes simplex virus; ICAM, intercellular adhesion molecule; MOI, multiplicity of infection; NF, nuclear factor; RV, rhinovirus.

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undertaken to determine whether RV has the ability to induce IL-6 production in vivo and in vitro and to characterize the mechanism(s) of the induction that was noted. These studies demonstrate that, in contrast to results among normals, IL-6 is detected immunologically and biologically in the nasal secretions of symptomatic patients with experimental RV upper respiratory tract infections, and that RVs are potent inducers of lung stromal cell IL-6 elaboration. They also demonstrate that RV stimulation of IL-6 production can occur in the presence and absence of intercellular adhesion molecule (ICAM) 1–mediated virus internalization, is associated with potent nuclear factor (NF)-κB activation, and is largely mediated by an NF-κB–dependent transcriptional activation process.

Methods

RV challenge of volunteers

The clinical study protocol that was used has been previously described (10, 18). In brief, healthy young adults 18 yr of age or older with reciprocal serum-neutralizing antibody titers of ≤ 2 to the challenge virus were recruited from the University of Virginia student body. Viral challenges were performed by administering twice in each nostril 0.25 ml vol of either RV type 39 or RV strain H (not neutralized by antisera to 89 numbered rhinovirus types, acid sensitive, and chloroform-idoxuridine resistant). A total dose of 800 50% tissue culture infectious dose ($TCID_{50}$) of RV strain H or 2,500 $TCID_{50}$ of RV type 39 were used. Starting on the morning before challenge and at 24-h intervals thereafter, all volunteers were interviewed regarding the presence and severity of the following 10 symptoms: sneezing, nasal discharge, nasal congestion, malaise, headache, chills, feverishness, sore throat, hoarseness, and cough. Symptoms were rated for severity on a scale from 0 to 3. Patients were designated as having a "cold" if they had a total symptom score of ≥ 5 over the 5 d after challenge plus either nasal discharge for 3 d or the belief that a cold had occurred (19).

Nasal lavages were performed with 10 ml of isotonic saline, once 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 −80°C until its IL-6 content was assayed. Serum samples were also obtained from some individuals at 24-h intervals on days 0–5. They were also stored at −80°C until assayed for IL-6. Viral culture was accomplished by combining lavage fluid with concentrated veal infusion broth and inoculating monolayers of MRC-5 human embryonic lung fibroblasts (BioWhittaker, Inc., Walkersville, MD). One isolate from each subject was identified as the challenge virus using a standard neutralization test. Homotypic neutralizing titers were determined by standard tests on blood collected before and 3 wk after inoculation (20). Volunteers were considered infected if they shed virus or had a fourfold or greater rise in serum antibody titer.

Viral stock preparation

RV14, RV1A, and herpes simplex virus (HSV) type 2 MS strain were 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 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 and 37°C for HSV-2. Infectivity of titers of stock viruses were determined by inoculation of serial dilutions into sensitive cell systems and the quantification of plaque formation as previously described by our laboratories (21).

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 rotor SW50.1 (model L5-50; Beckman Instruments, Inc., Palo Alto, CA). The resulting viral pellet was resuspended in NTE buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA) and 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 sterilized, viral titers were quantified via plaque assay, and purified virus was aliquoted and stored at −70°C until used.

Cell infection and supernatant preparation

MRC-5 human fetal lung fibroblasts and A549 alveolar epithelial type II–like cells were obtained from the ATCC and grown to confluence in 100-mm petri dishes in DME supplemented with nonessential amino acids, L-glutamine, penicillin, streptomycin, and 10% FBS (Hyclone Laboratories, Logan, UT). 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 PBS, DME with 2% FBS was introduced, and the cells were incubated at 33°C for up to 72 h. At designated time points, the supernatants were removed, clarified by low speed centrifugation, and stored at −70°C until used. The cell monolayers were then rinsed with PBS and used for mRNA analysis as described below.

IL-6 quantification

IL-6 was quantitated immunologically and biologically in these studies.

IL-6 ELISA. Immunoreactive IL-6 was quantitated using a dual antibody ELISA kit obtained from R&D Systems (Minneapolis, MN) following the manufacturer's protocol.

IL-6 bioassay. IL-6 bioactivity was assessed using modifications of the plasmacytoma proliferation assay previously described by this laboratory (22). In this assay, B9.11 cells (a gift from Dr. Mark C. Horowitz, Yale University, New Haven, CT) were incubated in round-bottomed microtiter wells at 5×10^3 cells/well in the presence and absence of recombinant IL-6–type cytokine standards and dilutions of the test fluids for 72 h. Cell proliferation was assessed by quantitating the [^{3}H]thymidine incorporation in each well during the final 16 h of this incubation period. Since both IL-11 and IL-6 can stimulate B9.11 cell proliferation (23), these assays were routinely run in the presence and absence of neutralizing antibodies against these cytokines. The anti–IL-6 antiserum was obtained from R&D Systems and was used at 7 μg/ml. mAb 11h3/19.6.1 was a gift of Drs. Edward Alderman and Paul Schendel (Genetics Institute, Cambridge, MA) and was used at 20 μg/ml.

mRNA isolation and analysis

Total cellular RNA was extracted from cell monolayers at desired time points using the acid-guanidinium isothiocyanate-phenol-chloroform extraction protocol described by Chomczynski and Sacchi (24). Equal amounts (10 μg) of RNA from each experimental condition were size fractionated by electrophoresis through 1% agarose, 17% formaldehyde gels, transferred to nylon membranes, and hybridized with ^{32}P-labeled plasmid cDNA probes. Clone pCSF-309, a full-length IL-6 cDNA, was a gift from Dr. Steven Clark (Genetics Institute). The probe was labeled to a high specific activity (10^9 cpm/μg DNA) using the random primer method (25), and its binding was assessed after washing under high-stringency conditions and autoradiography. The adequacy of gel loading was routinely assessed by ethidium bromide staining. Densitometry was performed using a scanning densitometer (GS 300; Hoefer Scientific Instruments, San Francisco, CA), and densitometry curve integration and analysis was performed with an appropriate program (GS370 Data System software package v3.0; Hoefer Scientific Instruments).

Nuclear run-on analysis

The relative rates of nuclear transcription were assessed using modifications of procedures previously described by this laboratory (26, 27).

Confluent cell monolayers (25×10^6 cells per sample) were incubated in the presence and absence of RV at 33°C for the noted period of time. The cells were then washed twice with PBS, mechanically detached, pelleted, and resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 3 μM DTT, 300 mM sucrose, 0.5% Triton X-100). The nuclei were then harvested by centrifugation and resuspended in 100 μl of storage buffer (50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, 40% glycerol) and stored at −70°C until further analyzed. Nylon membranes carrying 20 μg each of isolated cDNA fragments encoding IL-6 and GM-CSF and 3 μg of cDNA encoding 28S were prepared using a slot-blotting apparatus (MINIFOLD II, Schleicher and Schuell Inc., Keene, NH) and baked in a vacuum oven at 80°C for 2 h. In vitro transcription and RNA labeling was 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 incubation 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 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 incubating equal numbers of counts of radiolabeled RNA and DNA immobilized on nylon membranes as described above. The membranes were washed at high stringency, and binding was evaluated using autoradiography and densitometry.

Reporter gene constructs

The IL-6–luciferase constructs were generated by excising HindIII-SstI fragments containing wild-type or mutant IL-6 promoter sequences between −225 and +13 from chloramphenicol acetyltransferase constructs pIC225, pICmNF-κB, and pICmNF-IL6 (28, 29) and inserting them into pXP1 (ATCC) between the same sites. The resulting constructs were named pIL225, pILmNF-κB, and pILmNF-IL6, which contained wild-type, or mutated sequences derived from the IL-6 promoter.

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. (30). In this procedure, A549 cells were cultured until ~60% confluent in 60-mm petri dishes in complete DME with 10% FBS. They were then washed and gently agitated for 30 min at room temperature in the presence of DNA (4.5 μg) and 0.5 mg/ml DEAE-dextran in a volume of 300 μl. At the end of this incubation period, the cells were washed, virus or control solution was added, and the cells were incubated for 24 h at 33°C in 5% CO_2 and air. The cells were then washed with PBS, mechanically detached, pelleted, and resuspended in 0.25 M Tris-HCl, pH 7.8, in the presence of lysis reagent (Promega, Madison, WI). The lysates were then clarified by centrifugation and stored at −20°C. Luciferase activity was measured using the luciferase assay system from Promega. Quantification was obtained in a luminometer (model LB9501, Lumat, Bethold, Germany). Transfection efficiency was routinely controlled for by cotransfecting (1.5 μg) pCMV-β-gal (ATCC), a construct which 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. (31). 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 (EMSA)

Preparation of nuclear extracts. Nuclear extracts were prepared using modifications of the techniques of Schreiber et al. (32). 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, freshly supplemented with protease inhibitors (1 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethyl-sulfonyl fluoride), pelleted at 4°C, and resuspended and swelled in solution A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, with freshly added protease inhibitors as above) for 15 min on ice. Membrane lysis was accomplished by adding 25 μl of 10% NP-40 followed by vigorous agitation. The nuclei were then collected by centrifugation, resuspended in 50 μl of solution B (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and freshly added protease inhibitors as above), and agitated vigorously at 4°C for 15 min. The protein concentrations of the nuclear extracts were determined using the Bradford method (33), and the extracts were aliquoted and stored at −70°C until used.

Oligonucleotide probes. Four different NF-κB oligonucleotides were obtained commercially (Bio-Synthesis, Inc., Denten, TX) and used in the EMSA. They included the recognition site for classic NF-κB (5′-TCGACAGAGGGGACTTTCCGAGAGGC-3′), a mutant classic NF-κB (5′-TCGACAGAGAATACTTTCCGAGAGGC-3′), the IL-6 promoter NF-κB sequence (5′-TCGAAATGTGGGATTTTCCCATGAGT-3′), and a mutated IL-6 promoter NF-κB sequence (5′-TTTATCAAATGTAATATTTTCCCATGA-3′) (29). Oligonucleotide probe for activator protein (AP) 1 (5′-CGCTTGATGACTCAGCCGGAA-3′) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Electrophoresis. EMSAs were also performed using the techniques of Schreiber et al. (32). 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 Biolabs, Inc., Beverly, MA). The labeled probes were purified by push-column chromatography, diluted with buffer (10 mM Tris-HCl, pH 8.0, 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 1 h. Resolution was accomplished by electrophoresing 10 μl of the reaction solution (~ 10,000 cpm) on vertical 5 or 8% nondenaturing polyacrylamide gels containing 2% glycerol using TBE buffer (22.3 mM Tris-HCl, 22.3 mM boric acid, 0.25 mM EDTA, pH 8.0). Binding was assessed via autoradiography.

Supershift EMSA. Supershift assays were used to determine which members of the NF-κB family are involved in RV stimulation of IL-6 production. In these studies, EMSAs were performed as described above except that rabbit polyclonal antibodies against the NF-κB proteins p65, p50, p52, c-rel, and rel-B (Santa Cruz Biotechnology, Inc.) were included during the 1-h radiolabeled probe extract incubation period. Preimmune antiserum (Santa Cruz Biotechnology, Inc.) was routinely used to control for any nonspecific effects of these antisera.

Results

RV stimulation of IL-6 in vivo. To determine if IL-6 is present in vivo at sites of RV infection, nasal washings were obtained from 19 normal volunteers at baseline and at 24-h intervals after experimental RV inoculation. The IL-6 in these washings was evaluated by ELISA and compared with symptomatology as assessed by the patient's symptom score. At baseline, IL-6 was not detected in the nasal secretions of any of the volunteers. In contrast, immunoreactive IL-6 was readily detected in the washings from volunteers who developed colds after viral exposure. In these individuals, IL-6 was detected after as little as 24 h, peaked 48 h after viral inoculation, and decreased thereafter (Fig. 1). The amount of IL-6 that was noted appeared to be related to the severity of the reported virus-induced upper respiratory tract infection. This is based on the finding that patients with colds (symptom score = 27.1±3.9 [mean±SEM]) had higher levels of nasal IL-6 than volunteers who did not develop colds (symptom score = 3.6±1.1 [mean±SEM]) (Fig. 1) and the correlation between the magni-

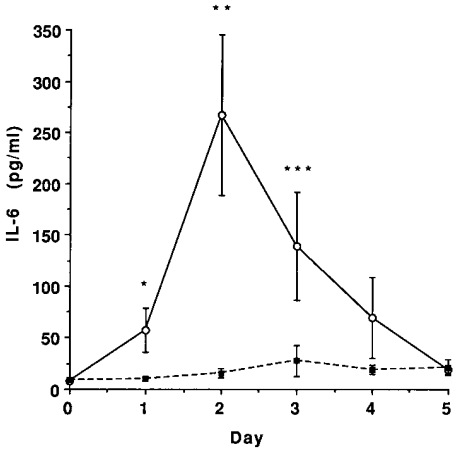

Figure 1. Immunoreactive IL-6 in nasal washings from patients with and without symptomatic upper respiratory tract infections after RV challenge. The IL-6 in the nasal washings of 19 different volunteers was quantitated before RV challenge (day 0) and at 24 h intervals thereafter. The levels of IL-6 in the washings from infected patients who developed colds ($n = 10$) (*solid line*) are compared with a group ($n = 9$) composed of patients who did not get infected ($n = 5$) or got infected but did not develop colds ($n = 4$) (*broken line*). *$P < 0.03$ comparing volunteers with colds on days 0 and 1; $P < 0.05$ comparing cold (+) and (−) patients on day-1 Student's *t* test. **$P < 0.004$ comparing volunteers with colds on days 0 and 2; $P < 0.008$ comparing cold (+) and (−) volunteers on day-2 Student's *t* test. ***$P < 0.03$ comparing volunteers with colds on day-0 and day-3 Student's *t* tests.

tude of the IL-6 response and patient symptom scores during the 5-d study period ($r = 0.8$, $P < 0.05$) (Fig. 2).

To determine if the IL-6 in the nasal washes from RV-infected individuals was biologically active, IL-6 bioactivity was assessed using the B9.11 plasmacytoma cell proliferation assay. Since these cells proliferate in response to IL-11 and IL-6, neutralizing antibodies to these moieties were routinely used to assess their relative contributions. None of the control nasal washings contained B9.11 proliferative activity. Similarly, significant IL-6 bioactivity was not detected over the 5-d study period in the washings from patients who did not develop colds after RV challenge (Fig. 3). In contrast, IL-6 bioactivity was readily detected in the nasal washings from patients who developed clinically significant colds. As seen at the protein level, this bioactivity could be appreciated after as little as 24 h, peaked 48–72 h after viral inoculation, and diminished thereafter (Fig. 3).

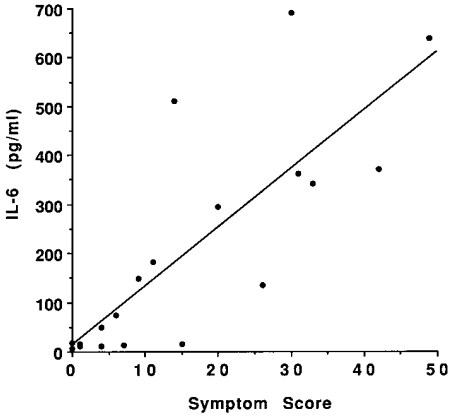

Figure 2. Correlation of the peak levels of nasal IL-6 and total symptom scores during the 5-d study period.

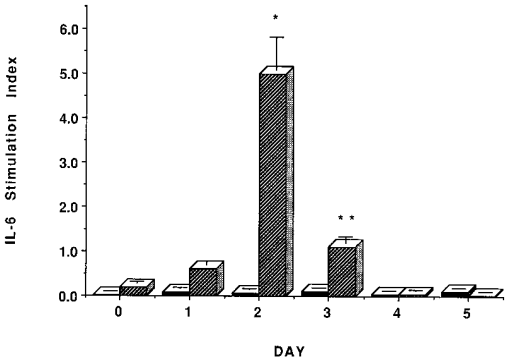

Figure 3. Bioactive IL-6 in nasal washings from RV-challenged patients. Nasal washings were obtained from RV-challenged individuals on the day before challenge (day 0) and at 24-h intervals thereafter. The levels of bioactive IL-6 in the fluids were determined using the B9.11 bioassay performed in the presence and absence of antibodies against IL-6 as described in Methods. The stimulation of B9.11 cell [^{3}H]thymidine incorporation that could be attributed to IL-6 is expressed on the y-axis as a stimulation index after being divided by the [^{3}H]thymidine incorporation of unstimulated control cells. Patients who developed colds are illustrated with the hatched columns and patients who did not with solid columns. Values represent the mean±SEM of evaluations of five randomly selected individuals in each group. *$P < 0.001$, Student's *t* test. **$P < 0.01$, Student's *t* test.

The IL-6 that was detected in the nasal washings could be locally produced. Alternatively, it could enter the nose from the circulation as a result of the permeability alterations induced by RV. To differentiate between these possibilities, we simultaneously collected sera and nasal washings from RV-challenged volunteers and compared their IL-6 content. As can be seen in Table I, IL-6 was readily detected in the nasal samples but not detected in the serum samples. This suggests that IL-6 production in RV-infected tissues is a localized phenomenon.

RV stimulation of IL-6 protein production and mRNA accumulation in vitro. To establish an in vitro system that would allow us to investigate the relationship between RV and IL-6, studies were undertaken to determine if RV stimulates lung cell IL-6 production in vitro. Unstimulated A549 cells did not produce IL-6, and MRC-5 cells produced small amounts of IL-6 constitutively. RV infection caused impressive increases in the IL-6 production of both cell lines. This stimulation could be appreciated within 4–8 h of infection and was even more

Table I. IL-6 in Nasal Washes and Serum

		Days after RV challenge*					
Patient	Sample	0	1	2	3	4	5
1	Nasal washes	4.60	11.2	341.11	48.10	8.50	7.70
	Serum	5.66	9.15	11.51	9.60	8.28	9.49
2	Nasal washes	7.70	188.80	295.40	21.30	9.00	7.00
	Serum	5.47	9.27	6.43	6.39	4.98	6.83

*IL-6 concentration (pg/ml). Values represent the mean of duplicates all of which were within 10% of each other.

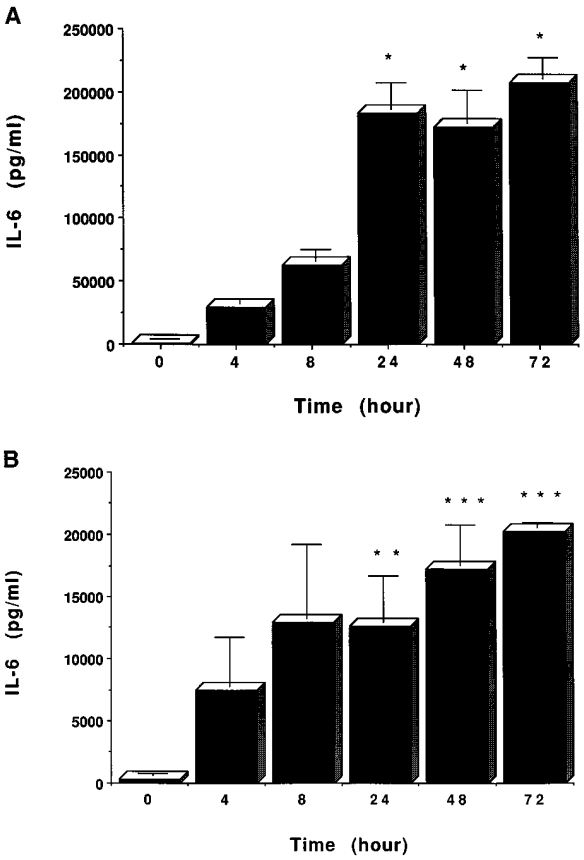

Figure 4. Kinetics of IL-6 elaboration by RV-infected lung cells. Monolayers of MRC-5 fibroblasts (*A*) and A549 lung epithelial cells (*B*) were infected with RV14 (MOI = 3), and the IL-6 in the cell supernatants was quantitated at intervals thereafter. $*P < 0.001$, $**P < 0.05$, and $***P < 0.01$ compared with unstimulated controls, Student's *t* test.

impressive after a 24–48-h incubation (Fig. 4). This induction appeared to be, to great extent, virus mediated, since purified RV14 had a similar ability to stimulate IL-6 production (data not shown), UV exposure markedly diminished the inductive capacity of RV14 (Fig. 5), and IL-6 production by fibroblasts was not similarly stimulated by endotoxin (data not shown). It was also at least partially virus specific, because HSV-2, at a similar MOI, did not have similar ability to stimulate IL-6 production (data not shown).

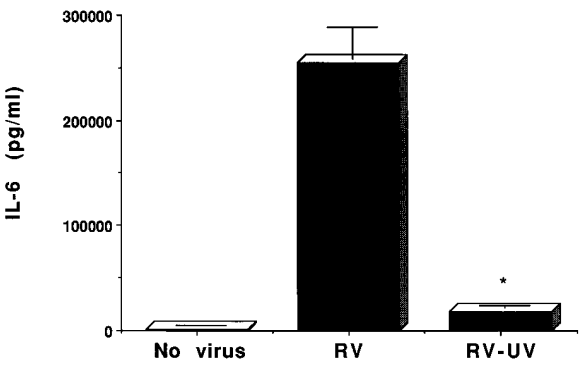

Figure 5. Demonstration of the effect of UV inactivation on RV14 stimulation of IL-6 production. MRC-5 lung fibroblasts were infected with equal quantities of viral stock solution. RV14 was used before and after UV light exposure (1,440 $\mu W/cm^2$ for 30 min, a dose that eliminates the ability of RV to generate cytopathic effects). IL-6 levels were determined after a 24-h incubation. $*P < 0.002$ compared with RV14 infected cells, Student's *t* test.

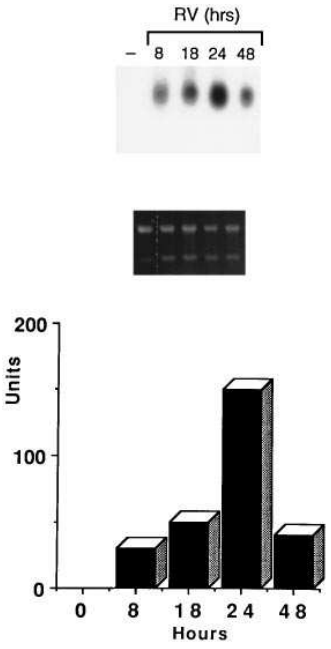

Figure 6. RV stimulation of IL-6 mRNA in MRC-5 lung fibroblasts. MRC-5 cells were infected with RV14 at an MOI of 3. After adsorption, the cells were rinsed and incubated in maintenance medium with 2% FCS. The levels of IL-6 mRNA in these cells at various time points after infection were evaluated as described in Methods. The top panel illustrates the effects of RV on IL-6 mRNA accumulation. Ethidium bromide loading controls are in the center and the densitometric analysis of the mRNA is illustrated at the bottom of the figure.

Unstimulated MRC-5 and A549 cells did not contain detectable levels of IL-6 mRNA. In accord with our findings with IL-6 protein, RV infection caused an impressive increase in IL-6 mRNA accumulation. This induction was time dependent and peaked 24 h after viral inoculation (Fig. 6 and data not shown). These studies clearly demonstrate that RV induction of IL-6 protein production in vitro is associated with a parallel increase in IL-6 mRNA and is, in great extent, pretranslationally mediated.

RV1A stimulation of IL-6 production. The majority of RVs, including RV14, bind to and enter cells using ICAM-1 (34). Approximately 10% of numbered serotypes, including RV1A, are in the minor receptor group and use the LDL receptor and related proteins for cell entry (35). To see if ICAM-1–mediated viral entry is required for RV induction of IL-6 production, we compared the stimulatory properties of RV14 and RV1A. As shown in Table II, RV14 and RV1A were similarly effective in stimulating IL-6 production. This demonstrates that RV can stimulate lung cell IL-6 production in the absence of ICAM-1–mediated virus internalization.

Table II. RV14 and RV1A Stimulation of IL-6

Virus*	IL-6
	pg/ml
None	1,109±108
RV14	188,259±21,990
RV1A	131,940±13,250

*MRC-5 fibroblasts were uninfected (*None*) or infected with RV14 or RV1A at an MOI of 3 and incubated for 48 h. The IL-6 in the resulting supernatants was assayed by ELISA.

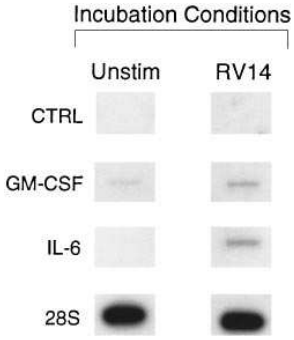

Figure 7. Effect of RV14 on IL-6 gene transcription. Nuclei were obtained from uninfected and RV14-infected lung cells 24 h after viral inoculation, and the rates of IL-6, GM-CSF, and 28S gene transcription were ascertained as described in Methods.

RV stimulation of IL-6 gene transcription. To further characterize the mechanism by which RV stimulates IL-6 production, nuclear run-on assays were performed to characterize the transcriptional effects of the virus. As can be seen in Fig. 7, IL-6 gene transcription was barely appreciated in nuclei from uninfected cells and readily apparent in nuclei of infected cells. This demonstrates that RV stimulation of lung cell IL-6 production is, at least partially, transcriptionally mediated.

RV stimulation of IL-6 promoter activity. Since transcriptional mechanisms play an important role in RV stimulation of IL-6 production, studies were initiated to define the *cis*-element(s) in the IL-6 promoter responsible for these effects. IL-6 promoter–luciferase reporter gene constructs were prepared and transfected into stromal cell monolayers. The luciferase activity in uninfected and RV-infected cells was then assessed. As seen in Fig. 8, luciferase was barely detected in uninfected cells. In contrast, construct pIL225, which contains the sequences in the IL-6 promoter between −225 and +13, responded briskly to RV14. Mutation of the NF-IL-6 site (mNF-IL-6) in this construct caused a ∼ 40% decrease in luciferase activity. In contrast, mutation of the NF-κB site (mNF-κB) decreased the response of pIL225 to RV14 by ∼ 90–95%. These studies clearly demonstrate that RV response elements are present in the region between −225 and +13 in the IL-6 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-6 promoter activity.

RV induction of NF-κB. To further understand the NF-κB–dependent mechanism(s) by which RV stimulates IL-6 promoter activity, studies were undertaken to determine if RV could alter NF-κB binding activity in nuclei from human lung cells. Nuclei from uninfected and infected cells were isolated, lysates were prepared, and EMSAs were performed using labeled wild-type and mutated IL-6 promoter NF-κB and classic NF-κB sequences. NF-κB binding was constitutively present in nuclei from uninfected cells and appeared as three bands on EMSA (Figs. 9 and 10). RV infection did not significantly alter this binding. It did, however, induce additional NF-κB binding activity that migrated as two new bands on 5% gels (Figs. 9 and 10) and a single broad band on 8% gels (Fig. 11). This RV-inductive effect did not require protein synthesis (Fig. 9) and had a distinct kinetic being readily appreciated 0.5 h after infection and decreasing with longer incubations (Fig. 10). It also appeared to be at least partially NF-κB specific, since AP-1–DNA binding was not similarly altered (Fig. 10). Rel/NF-κB proteins appeared to mediate this RV-induced binding, since unlabeled IL-6 promoter NF-κB sequences and classic NF-κB sequences effectively competed whereas mutated IL-6 promoter NF-κB sequences did not compete for DNA binding (Fig. 11). Supershift EMSA also demonstrated that antibodies to p65 completely abrogated and antibodies to p50 or p52 significantly diminished both RV-induced NF-κB bands (Fig. 12). In contrast, antibodies to c-rel, rel-B, and preimmune serum did not significantly alter RV-induced NF-κB binding (Fig. 12). These studies demonstrate that RV selectively induces NF-κB binding in lung stromal cells and that p65 is the common constituent of these NF-κB complexes.

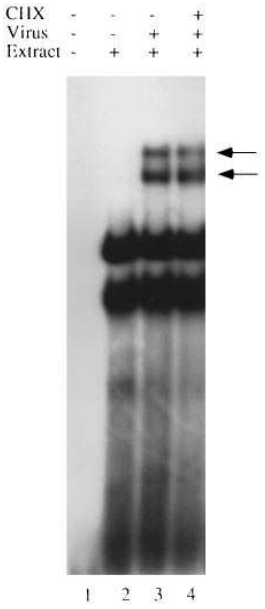

Figure 9. Role of protein synthesis in RV14 stimulation of NF-κB binding activity. Nuclear lysates were prepared from uninfected and RV14-infected A549 cells, and EMSAs were performed using 5% polyacrylamide gels as described in Methods. Radiolabeled IL-6 NF-κB sequences were used in the hybridization mixture. RV induction of NF-κB binding activity was assessed in the presence and absence of cycloheximide (25 μg/ml). The RV-inducible NF-κB binding activities are highlighted with the arrows.

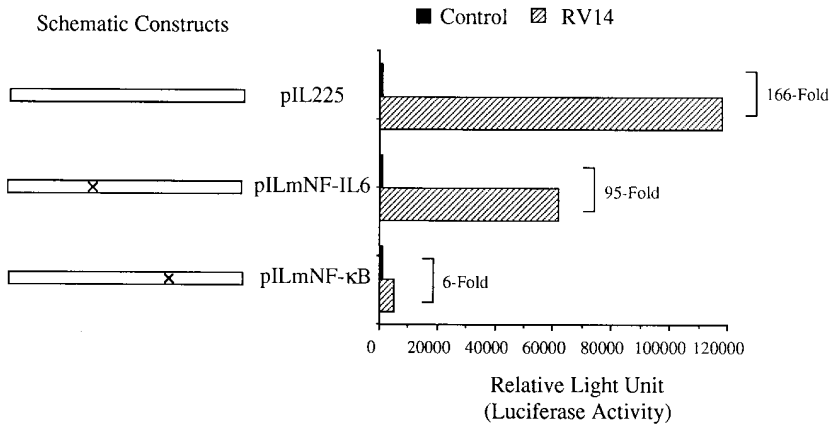

Figure 8. IL-6 promoter-driven luciferase activity in A549 cells. A549 cells were transfected with IL-6 promoter–luciferase constructs and then incubated under control conditions or infected with RV (MOI = 3). Uninfected and RV-infected cells were incubated for 24 h, cell layers were harvested, and luciferase activity was assessed as described in Methods. β-Galactosidase activity was also assessed, and these determinations were used to correct for transfection efficiency. The luciferase activity in uninfected cells (control) and RV14-infected cells containing constructs pIL225, pILmNF-IL-6, and pILmNF-κB are illustrated.

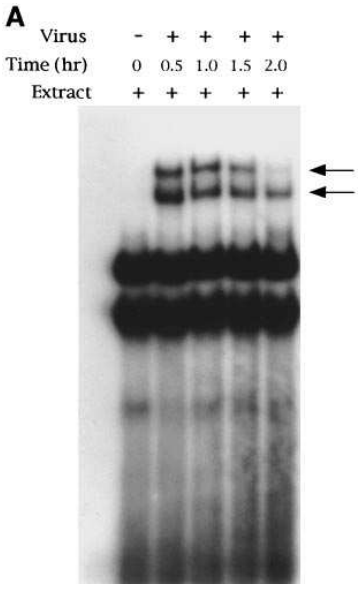

Figure 10. Kinetics of RV stimulation of DNA binding activity in A549 cells. Nuclear lysates were prepared from uninfected and RV14-infected cells at varying times after viral inoculation. The NF-κB binding activity in these lysates was evaluated by EMSA using 5% polyacrylamide gels as described in Methods. Radiolabeled IL-6 NF-κB sequences (*A*) and AP-1 sequences (*B*) were used in the hybridization mixture. The two inducible NF-κB binding activities are highlighted by the arrows.

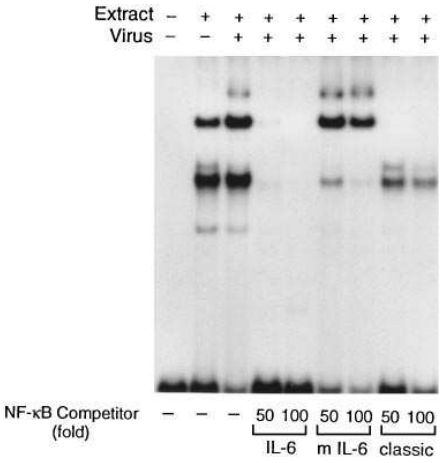

Figure 11. Specificity of RV14-induced NF-κB binding activity. Nuclear lysates were prepared from uninfected and RV14-infected A549 cells. The NF-κB binding activity in these lysates was assessed using radiolabeled IL-6 NF-κB sequences in the presence and absence of unlabeled IL-6 NF-κB motifs (*IL-6*), mutated IL-6 NF-κB motifs (*mIL-6*), and classic NF-κB motifs (*classic*). Resolution was accomplished on 8% gels as described in Methods.

Discussion

Viruses mediate their pathologic effects, in great extent, via local cytopathology and the induction of tissue inflammation. Studies of RV respiratory tract infections using organ cultures (7, 36), punch biopsies (8), scrape biopsies (6), and nasal secretions (37) have demonstrated, however, that cell cytotoxicity is not a major contributor to human RV pathologies. As a result, it is now believed that the immunologic or inflammatory responses of the host to RV infection are of primary importance in the production of its clinical and symptomatic abnormalities (38). To further understand the cellular and molecular events involved in the host response to RV, studies were undertaken to determine if IL-6 could be detected at sites of RV infection. In vitro approaches were also used to determine if RV stimulates lung cell IL-6 production and to characterize the mechanism of the stimulation that was noted. These studies demonstrate that, in contrast to normals, impressive levels of IL-6 are present in the nasal secretions of symptomatic volunteers 48–72 h after nasal RV inoculation and that this IL-6 is locally produced. They also demonstrate that RVs are potent stimulators of lung fibroblast and epithelial-like cell IL-6 elaboration, that this stimulation can occur after ICAM-1–mediated or non–ICAM-1–mediated virus internalization, that RV is a potent activator of NF-κB, and that RV-stimulated IL-6 production is mediated, in great extent, via an NF-κB–dependent transcriptional activation process. These studies are the first to document dysregulation of IL-6 at sites of RV infection. They are also the first to define the mechanism by which RV stimulates cytokine production and the first to demonstrate that RV has the ability to activate members of the ubiquitous Rel/NF-κB transcription factor family.

Early descriptions of IL-6 focused on its antiviral activity and referred to IL-6 as IFN-β_2 (39). This antiviral activity has subsequently proven to be limited. In contrast, the immunomodulatory effects of IL-6 have proven to be profound. Our studies demonstrate that IL-6 is present in exaggerated quanti-

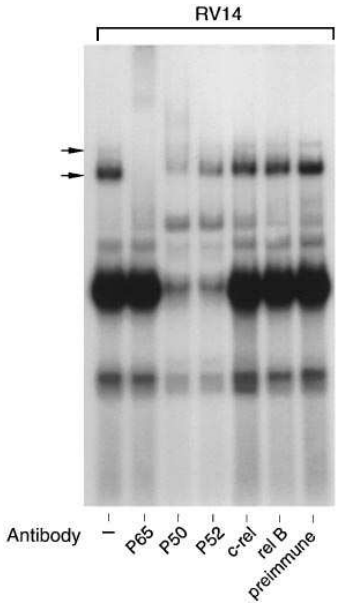

Figure 12. Identification of NF-κB moieties using supershift EMSA. Nuclear lysates were prepared from RV14-infected A549 cells, and EMSAs were performed in the presence and absence of antisera against NF-κB family proteins. Radiolabeled IL-6 NF-κB oligonucleotides were used in the hybridization mixture. Antisera against p65, p50, p52, c-rel, and rel-B are compared with preimmune serum. RV14-induced NF-κB binding activities are highlighted by arrows.

ties in the nasal lavage fluids of normal volunteers with RV-induced symptomatic upper respiratory tract infections. These observations are in accord with studies of naturally occurring upper respiratory tract infections in children (in which viral etiology was not determined) (40) and support the contention that dysregulated IL-6 production is a prominent feature of RV infection. These observations suggest that IL-6 is involved in the generation of the symptomatology and/or the host response to RV infection. It is impossible to state with certainty, however, what role IL-6 is playing in this disorder. Potentially important in vitro effects of IL-6 include its ability to act as an endogenous pyrogen (41), stimulate the acute phase response (42), stimulate T lymphocytes (43), and induce the terminal differentiation and Ig production of B lymphocytes (44). If similar effects are operative in vivo, IL-6 could contribute to the fever, systemic symptomatology, submucosal lymphocyte infiltration, and antiviral Ig response seen after RV infection (1, 45). The latter may be particularly important since mice with targeted disruptions of the IL-6 gene have deficient IgA responses after viral challenge (15), and IgA plays an important role in RV immunity (1).

The mechanism(s) by which RV stimulates lung stromal cell IL-6 production is poorly understood. This process may, however, be initiated by virus binding to specific cell surface viral receptors. Approximately 85–90% of RVs (the major receptor group) bind to and enter cells using the membrane protein ICAM-1 (34). Almost all of the remaining RVs (the minor receptor group) use the LDL receptor and related proteins (35). To determine if RV binding to ICAM-1 (or the LDL receptor) is required for IL-6 elaboration, we compared the IL-6 stimulation induced by RV14 and RV1A, which are major and minor receptor group viruses, respectively. Our studies demonstrate that both viruses are potent stimulators of IL-6 elaboration. This demonstrates that RV stimulation of IL-6 elaboration occurs after RV binding and internalization via ICAM-1 or the LDL receptor complex. If RV14 and RV1A stimulate IL-6 production via common mechanisms, these commonalities exist at a postreceptor level.

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 life-saving immune and inflammatory events. In the case of viruses, the infecting agent must be recognized as foreign and the necessary antimicrobial defenses must be marshaled at the relevant location. A major step in this response is the triggering of the cytokine cascade with the production of a designated repertoire of cytokines by local immune and nonimmune cells. The Rel/NF-κB transcription factor family stands out as a central coordinator of this and other responses to stress and injury (46). Under most circumstances, these factors lay dormant in the cytoplasm, kept there by inhibitory IκB proteins. They dissociate from IκB in response to a wide variety of activating stimuli, including cytokines, mitogens, physical stresses, oxidative stresses, chemical agents, bacteria, and a variety of viruses. They then enter the nucleus and regulate the transcription of a variety of genes involved in inflammation, immunity, and stress responses via the formation of *trans*-activating dimers amongst themselves and by interacting with other transcription factors (for review see reference 46). Our studies demonstrate for the first time that RV is a potent activator of NF-κB DNA binding, and that NF-κB activation plays an important role in RV stimulation of IL-6 elaboration. These findings are in accord with the activation of NF-κB by a number of other respiratory viruses including cytomegalovirus, Sendai virus, and adenovirus (for review see reference 46). These findings are also in agreement with previous observations from our laboratories and others demonstrating that NF-κB plays a crucial role in the activation of IL-6 gene transcription in response to noninfectious stimuli such as IL-1 (28, 29, 47). The demonstration that RV is a potent activator of NF-κB has important implications, since a variety of other NF-κB–responsive genes may also play important roles in the pathogenesis of RV pathologies. They include IL-2, which could contribute to the submucosal lymphocyte collections seen after RV infection (45); IL-8, a chemokine that could be responsible for the tissue neutrophilia seen during the common cold (38); ICAM-1, the major RV receptor (34); and IFN-regulatory factors 1 and 2 (46). In recent years, it has become clear that NF-κB activation also plays a role in the regulation of the replication of a number of viruses including cytomegalovirus (48, 49) and HIV-1 (50, 51). Our studies do not address the role, if any, that NF-κB plays in regulating RV replication. They do, however, raise the possibility that RV-induced NF-κB activation can modulate the expression of other NF-κB regulated viruses that may be simultaneously present in infected tissues.

The NF-κB–like motif GGGATTTCC in the IL-6 promoter is recognized by p50 homodimers, p50-p65 heterodimers, and p65 homodimers in in vitro binding assays (47). To determine the relative contribution that each of these dimers makes to RV induction of IL-6 elaboration, supershift EMSAs were performed. Our studies demonstrate that antibodies against p65 completely abolish RV-induced NF-κB binding. Antibodies against p50 and p52 also caused modest decrements, whereas antisera against c-rel and rel-B did not alter RV-induced NF-κB binding. These observations suggest that the p65 subunit of NF-κB is the common component of RV-induced NF-κB complexes, which include both p50-p65 and p52-p65 heterodimers. It is important to note, however, 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 (46, 52). For example, we and others have shown the requirement for both the NF-κB and the NF-IL-6 sites for induction of the IL-6 promoter by IL-1 (29, 47). Direct association between these transcription factors has also been established (53). In this manuscript, we have demonstrated that mutation of the NF-IL-6 site in the IL-6 promoter modestly decreases its responsiveness to RV. This suggests that NF-IL-6 plays a role in RV induction of IL-6 elaboration. It also raises the possibility that maximal RV-induced IL-6 elaboration depends on NF-κB–NF-IL-6 interactions.

In a number of systems, viral activation of target gene transcription is mediated by virus-encoded *trans*-activating proteins that act at least partially through NF-κB. Prominent examples of this include the activation of IL-6 by the human T cell leukemia virus type I tax gene (54) and the X product of hepatitis B virus (55). Our studies demonstrate that RV stimulates IL-6 production and activates NF-κB in lung cells. This raises the possibility that RV contains *trans*-activating proteins that activate IL-6 via similar pathways. Additional investigation will be required to test this possibility.

RV infections are a major health care issue since, on average, adults experience 2–4 and children experience 4–10 infec-

tious episodes per year (56–58). Our studies demonstrate that NF-κB activation is prominently noted after RV infection and plays an important role in RV-induced cytokine production. In contrast, AP-1 DNA binding was not significantly altered. This suggests that NF-κB activation may be a central component in the pathogenesis of RV-induced pathologies. NF-κB activation is a complex process that involves IκB phosphorylation, ubiquitination, proteolytic digestion, and the movement of NF-κB into the nucleus (46, 59). In addition, cellular thiol levels and oxidant and antioxidant levels can regulate the activation process (46, 60). If NF-κB activation is a crucial event in the pathogenesis of RV infection, it becomes an attractive site upon which to base new therapies for this disorder. We have previously demonstrated that inhibition of IL-6 gene expression by glucocorticoids involves direct physical association between the glucocorticoid receptor and NF-κB (29). Aspirin and sodium salicylate have already been shown to decrease IκB degradation and NF-κB activation at high concentrations (61). NF-κB–inhibiting protease inhibitors (62), antioxidants (63), and antisense oligonucleotide p65 (64) are examples of potential therapeutic approaches that would warrant further investigation.

In summary, these studies demonstrate that the levels of immunoreactive and bioactive IL-6 are significantly increased in the nasal secretions of volunteers experiencing symptomatic RV infections compared with those obtained from normal volunteers and challenged minimally symptomatic controls. These studies also demonstrate that RV is a potent stimulator of IL-6 elaboration by lung stromal cells and demonstrate that this stimulatory process is not dependent on ICAM-I virus internalization, is associated with NF-κB activation, and is mediated by an NF-κB–dependent transcriptional activation pathway. They suggest that IL-6 plays an important role in the pathogenesis of RV infection and that NF-κB activation may be an important event in RV-induced pathologies.

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