Rhinovirus Infection Induces Expression of Its Own Receptor Intercellular Adhesion Molecule 1 (ICAM-1) via Increased NF-κB-mediated Transcription*

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Virus infections, the majority of which are rhinovirus infections, are the major cause of asthma exacerbations. Treatment is unsatisfactory, and the pathogenesis unclear. Lower airway lymphocyte and eosinophil recruitment and activation are strongly implicated, but the mechanisms regulating these processes are unknown. Intercellular adhesion molecule-1 (ICAM-1) has a central role in inflammatory cell recruitment to the airways in asthma and is the cellular receptor for 90% of rhinoviruses. We hypothesized that rhinovirus infection of lower airway epithelium might induce ICAM-1 expression, promoting both inflammatory cell infiltration and rhinovirus infection. We therefore investigated the effect of rhinovirus infection on respiratory epithelial cell ICAM-1 expression and regulation to identify new targets for treatment of virus-induced asthma exacerbations. We observed that rhinovirus infection of primary bronchial epithelial cells and the A549 respiratory epithelial cell line increased ICAM-1 cell surface expression over 12- and 3-fold, respectively. We then investigated the mechanisms of this induction in A549 cells and observed rhinovirus-induction of ICAM-1 promoter activity and ICAM-1 mRNA transcription. Rhinovirus induction of ICAM-1 promoter activity was critically dependent upon up-regulation of NF-κB proteins binding to the −187/-178 NF-κB binding site on the ICAM-1 promoter. The principal components of the rhinovirus-induced binding proteins were NF-κB p65 homoeodimers. These studies identify ICAM-1 and NF-κB as new targets for the development of therapeutic interventions for virus-induced asthma exacerbations.

Asthma is increasingly common and now affects up to 30% of the population in westernized countries (1). Asthma exacerbations are the major cause of asthma morbidity and mortality. Respiratory viral infections have recently been associated with the majority of asthma exacerbations in both adults and children. In community-based studies, viral infections were identified in 80–85% of exacerbations in children and 44% of exacerbations in adults (2, 3). Viral infections have also been strongly implicated in more severe asthma exacerbations requiring hospitalization in both children and in adults (4). In all of these studies, rhinovirus infections caused around 65% of exacerbations in which a virus was identified (2–4). Rhinovirus-induced asthma exacerbations therefore cause enormous morbidity, especially among children, and represent a major health and economic problem. To date, no safe effective therapy is available, since prophylactic inhaled steroids are ineffective (5), while intervention with high dose inhaled steroids is only partially effective (6). A better understanding of the mechanisms involved in rhinovirus-induced asthma exacerbations would greatly aid the development of new therapies for this common condition.

The mechanisms by which rhinoviruses trigger asthma exacerbations are poorly understood. Asthma is an inflammatory disease of the lower respiratory airways, and lower airway inflammatory changes have been described during experimental rhinovirus colds. A marked bronchial CD3+, CD4+, and CD8+ T lymphocyte and eosinophil infiltration was observed in biopsies taken at the height of cold symptoms in both normal and in asthmatic subjects (7). However, the eosinophil infiltrate was more prolonged in the asthmatic subjects, still present 6–8 weeks after infection, while the eosinophil counts in normal subjects had returned to baseline (7). Rhinovirus experimental infections have also been reported to increase allergen-induced eosinophil numbers in bronchial lavage fluid in atopic rhinitic subjects, while no change in eosinophil numbers was observed in normal subjects (8), and to increase eosinophil products in sputum supernatants in asthmatic subjects (9). These data combined strongly suggest that rhinovirus-induced bronchial lymphocyte and eosinophil infiltration and activation are probably very important mechanisms in virus-induced asthma exacerbations.

The increased airway reactivity demonstrated in experimental rhinovirus infections in asthmatic subjects (9, 10) and atopic subjects (11) and the induction by rhinovirus of late asthmatic responses to inhaled allergen (8, 11) also provide indirect evidence of a link between lower respiratory inflammation during rhinovirus experimental infections and the mechanisms of virus-induced asthma exacerbations. Finally, evidence that the lymphocytic and eosinophilic inflammation observed during rhinovirus experimental infections (7–9) is probably also an important mechanism involved in virus-induced asthma exacerbations comes from the fact that asthma exacerbations have been induced by experimental rhinovirus infections (9, 12).

Rhinovirus RNA has recently been detected in bronchial lavage cells taken during experimentally induced colds, suggesting that rhinovirus can promote local inflammation by direct infection of the lower airways (13). Indeed, rhinoviruses are capable of prolonged, noncytolytic infection of respiratory epithelial cells and induce production of proinflammatory cy-
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MATERIALS AND METHODS

Cell Culture

Rhinovirus types 16, 9 (major group), and 2 (minor group) were obtained from the American Type Culture Collection (ATCC; Rockville, MD), and Ohio HeLa cells were obtained from the Medical Research Council Common Cold Unit (Salisbury, UK). Cells were split weekly and cultured at 37 °C in 5% carbon dioxide in Eagle's minimal essential medium supplemented with 4 mM l-glutamine, 80 mg/ml of gentamycin, and 10% fetal bovine serum (Sigma, Poole, UK). Primary human bronchial epithelial cells were obtained by bronchial brushing from normal patients undergoing surgery. Cells were removed from the brush by vigorous shaking and were disaggregated in Clonetics (San Diego, CA) bronchial epithelial cell medium containing 3 mM DTT for 15 min. After washing, cells were plated onto collagen-coated 16-mm diameter culture wells and grown to confluence in bronchial epithelial cell medium. Cells were passaged in 100-mm Petri dishes and used in the assays at passages 3 and 4. For experiments, 70% confluent cells were detached using 0.05% trypsin with 0.02% EDTA and seeded at 2 x 10^5 cells/well in 12-well culture plates. These cells are >95% cytokeratin 18-immunoreactive epithelial cells as assessed by immunofluorescence microscopy.

Viral Stocks

Rhinovirus types 16, 9 (major group), and 2 (minor group) were obtained from the Medical Research Council Common Cold Unit, and their identity was confirmed by neutralization with specific antiserum (ATCC). Viral stocks were generated by infecting monolayer cultures of HeLa cells until cytopathic effects were fully developed. Cells and supernatants were harvested, cells were disrupted by freezing and thawing, cell debris was pelleted by low speed centrifugation, and the resulting clarified supernatants were frozen at ~70 °C.

Rhinovirus titration was performed on the frozen aliquots by exposing confluent monolayers of HeLa cells in 96-well plates to serial 10-fold dilutions of viral stock. Plates were cultured for 5 days in 4% minimal essential medium at 37 °C in 5% CO₂. Cytopathic effect was assessed by visual assessment and by assessment of the continuity of the monolayer after fixation in methanol and staining with 0.1% crystal violet. Tissue culture infective dose 50% (TCID₅₀)/ml values were determined (28), and virus at a multiplicity of infection (MOI) of 1 was used for all of the experiments except where indicated.

Rhinovirus Inactivation

For selected experiments, inactivation/filtration of the virus was performed by three different methods.

Prevention of Virus-Receptor Binding

Viruses were precoated with excess soluble receptor to saturate the receptor binding sites on the virus capsid. Virus stock solutions were preincubated with recombinant soluble ICAM-1 (sICAM; a gift of P. Esmen, Bayer Corp., Berkeley, CA) at a concentration of 1 mg/ml for 30 min at room temperature.

Prevention of Virus Replication

Viruses were inactivated by exposure to UV light at 1200 mJ/cm² for 30 min.

Filtration of Virus from Inoculum

Virus particles were removed from inocula by ultrafiltration through membranes (Amikon, London, UK) to remove all molecules greater than 30 kDa, performed according to the manufacturer’s instructions.

For each method, confirmation of complete inactivation was carried out by microtiter plate assay for rhinovirus infectivity as described above.

Measurement of ICAM-1 Surface Protein Expression

2 x 10^5 A549 or primary bronchial epithelial cells were cultured in 12-well plates. When confluent, virus at an MOI of 1 or control media was added, and incubation continued for various periods of time between 1 and 72 h. Dose-response studies were carried out using 0.05, 0.1, 0.5, 1, and 2 MOI, and cells were harvested at 8 h. Similarly, the

1 The abbreviations used are: IL, interleukin; ICAM-1, intercellular adhesion molecule-1; sICAM-1, soluble ICAM-1; TCID₅₀, tissue culture infective dose 50%; APRT, adenine phosphoribosyltransferase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; MOI, multiplicity of infection; DTT, dithiothreitol; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; bp, base pair(s); C/EBP, CCAAT/enhancer element-binding protein.
effects of inactivated/filtered virus and the effects on primary bronchial epithelial cells were studied at 8 h. At the desired time points, cells were detached intact by incubation with 0.5 ml/well cell dissociation solution (Sigma) at 37 °C for 10 min. More than 95% of cells were viable as determined by trypan blue dye exclusion. 10⁶ cells were then washed and resuspended in PBA (phosphate-buffered saline, 1% bovine serum albumin, 0.1% sodium azide) and incubated with saturating amounts of fluorescein isothiocyanate-conjugated anti-human ICAM-1 (CD54) antibody or isotype-specific control antibody (Serotec, Oxford, UK) for 30 min at 4 °C in the dark. After washing, 10⁶ cells were analyzed for fluorescence by single color flow cytometry on a FACScan analyzer (Becton Dickinson, San Jose, CA). Mean fluorescence intensity was measured and normalized relative to noninfected control cells after subtraction of background staining.

**ICAM-1 mRNA Analysis**

5 × 10⁶ A549 cells were cultured in 100-mm plates until confluent, and medium alone or rhinovirus type 16 was added for various times between 1 and 24 h. Studies with inactivated/filtered virus were performed at 8 h. At the desired time points, cells were harvested, and ICAM-1 mRNA expression was evaluated by RT-PCR. Whole cell RNA was extracted using Trizol according to the manufacturer's instructions (Life Technologies, Inc., Paisley, UK). One microgram of total RNA was reverse transcribed by superscript reverse transcriptase (100 units; Promega, Madison, WI) in a final volume of 10 μl at 37 °C for 1 h using P1 (24 ng/ml) as specific primer (Table I). The cDNA (2.5 μl) was amplified by PCR in the presence of a master mix containing PCR buffer, MgCl₂ (1.5 mM), 1.25 units of taq DNA polymerase (Promega), 0.2 mM dNTPs, and 0.6 mM specific primer pair (P1 and P2; Table I). Cycling conditions were 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C for 25 cycles. First-round products were diluted 1:100, and 2.5 μl was thereafter used for a nested amplification under the same PCR conditions, with P3 and P4 as inner primers (Table I). Final PCR products (10 μl) were electrophoresed through 1.5% agarose gels, stained in ethidium bromide, and photographed under UV light. In parallel, mRNA for adenine phosphoribosyltransferase (APRT), using primers indicated in Table I for 40 cycles at 56 °C, was evaluated in each sample as housekeeping gene ribosyltransferase (APRT), using primers indicated in Table I for 40 cycles at 56 °C, was evaluated in each sample as housekeeping gene.

**ICAM-1 Nuclear Transcription Analysis**

Isolation of nuclei and in vitro nuclear transcription were performed using standard procedures (29). Confluent cell monolayers (2.5 × 10⁷ cells) were incubated with medium alone or rhinovirus 16 for 1 h. Cells were then washed twice with phosphate-buffered saline, harvested, and centrifuged at 500 × g for 5 min. The cell pellet was resuspended in 1 ml of 10 mM Tris-HCl, pH 8.4, 1.5 mM MgCl₂, 0.14 mM NaCl and lysed by the addition of 5% Nonidet P-40 followed by vigorous agitation and photographed under UV light. In parallel, mRNA for adenine phosphoribosyltransferase (APRT), using primers indicated in Table I for 40 cycles at 56 °C, was evaluated in each sample as housekeeping gene control. Densitometry was performed using a scanning densitometer, and densitometric analysis was performed using the Phoretix program (Bioimaging Ltd., Newcastle-upon-Tyne, UK) to express ICAM-1 mRNA relative to APRT mRNA.

To confirm the quantitative nature of the PCR, cell lysate was diluted 3-fold in triplicate and subjected to RNA extraction, RT-PCR, and densitometric analysis.

**Cell Transfection and CAT Assay**

A549 cells were transfected with reporter constructs (10 μg) at 80% confluency by the calcium phosphate precipitation technique for 5 h, glycerol-shocked with 1× HeBS (0.02 mM Heps, 0.135 mM NaCl, 0.5 mM Na₂HPO₄, 5.5 mM d-glucose, pH 7.1), 15% glycerol for 30 and washed. Transfected cells were cultured in 10% minimal essential medium for 24 h, and rhinovirus 16 or medium alone was added. At designated time points, cells were harvested, and cell extracts were prepared by three cycles of rapid freeze-thawing in 0.25 M Tris, pH 8.0. Each sample was incubated at 65 °C for 15 min to inactivate endogenous transactetylases. Protein content was determined photometrically using the Bio-Rad protein assay (Bio-Rad). Protein-equivalent aliquots were assayed for CAT activity according to standard protocols (33). The assay was performed at 37 °C for 60 min in a reaction mixture of 1 μl acetyl-CoA (Amersham Pharmacia Biotech) and 0.1 μl DNA polymerase (Taq) containing wild-type and mutated sequences of ICAM-1 AP-1, NF-κB, and CREB, respectively. Acetylated and unacylated forms were resolved by thin layer chromatography, visualized by autoradiography, and measured on a scintillation counter. CAT activity was expressed as percentage of chloramphenicol converted to its acetylated derivatives.

**Electrophoretic Mobility Shift Assay (EMSA)**

**Preparation of Nuclear Extracts**—Uninfected and rhinovirus-infected A549 cells were prepared as described previously. At the desired time points (0, 30, 60, 90, and 120 min), the cells were mechanically detached, and nuclear extracts were obtained by a modification of the method of Dignam et al. (34). Briefly, after washing with phosphate-buffered saline, cells were centrifuged at 4 °C and resuspended in buffer A (10 mM Heps, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT) with freshly added protease inhibitors (10 μg/ml leupeptin, 5 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). Membrane lysis was achieved by adding 0.5% Nonidet P-40 followed by vigorous agitation and incubation on ice for 5 min. The nuclei were then pelleted at 4 °C and resuspended in buffer C (20 mM Heps, pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and freshly added protease inhibitors as above). This suspension was incubated on ice for 15 min and centrifuged. The protein concentration of the nuclear extracts was photometrically determined using the Bio-Rad protein assay.

**Oligonucleotide Probes (Table II)**—Double-stranded oligonucleotides containing wild-type and mutated sequences of ICAM-1 AP-1, NF-κB, Sp1, and CREB recognition sequences were obtained commercially (Oswell DNA Service, Southampston, UK). Mutant sequences were identical to those used in the mutant reporter constructs. Probes containing NF-κB, AP-1, or Sp1 consensus sequences were commercially obtained (Promega).

Oligonucleotides were end-labeled with [γ-³²P]ATP and T4 polynucleotide kinase (Promega). Equal amounts (5 μg) of nuclear protein were incubated with 10 fmol of probe in binding buffer (10 mM Tris, pH 7.5, 5% glycerol, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, and 1.25 μg of poly(dI-dC)poly(dI-dC)) for 30 min at room temperature. Complexes were resolved on 5% nondenaturing polyacrylamide gels in TBE buffer (50 mM Tris, pH 8.0, 50 mM boric acid, 1 mM EDTA) containing 4% glycerol. Electrophoresis was performed at 10 V/cm for 2–3 h. Gels were dried, and binding was assessed by autoradiography.

**Supershift EMSA**—Supershift assays were used to study which members of the NF-κB family were involved in rhinovirus-induced formation of complexes with the ICAM-1 promoter sequence –199/–170. One μl of rabbit polyclonal antibodies against each of p65, p50, p52, c-Rel, and Rel-B (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to 2 μg of nuclear extracts for 15 min at 4 °C before the incubation with radiolabeled probe as described above. Rabbit preimmune serum was used as negative control.
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**TABLE II**
Double-stranded oligonucleotides used in electrophoretic mobility shift assays

| Sequence of double-stranded oligonucleotides | Position in ICAM-1 promoter | Type of binding site | Position of binding motif | Mutated binding site |
|---------------------------------------------|-----------------------------|---------------------|--------------------------|---------------------|
| 5′-GGGTGACATCGCCCTGAGTTTCCGTGA             | −294/−266                   | AP-1                | −284/−278                |                     |
| 5′-CCGACTTTAGCTGGAATTCGCGGACGTGA           | −199/−170                   | NF-κB               | −199/−196                | C/EBP (M)           |
| 5′-ATGCTTCTAGCTGGAATTCGCGGACGTGA           | −199/−170                   | NF-κB               | −199/−196                | NF-κB (M)           |
| 5′-CTGGAAACCGACCCGCCCCTGCCCCCA            | −74/−43                     | Sp1                 | −59/−54                  |                     |

**RESULTS**

Rhinovirus Induces ICAM-1 Cell Surface Protein Expression in A549 Cells and in Primary Bronchial Epithelial Cells—Preliminary studies indicated that rhinovirus infection of A549 cells up-regulated ICAM-1 surface expression at 8 h postincubation. Dose-response studies in A549 cells exposed to rhinovirus 16 were therefore carried out to determine if the induction of ICAM-1 occurred in a dose-response manner. Cell surface ICAM-1 expression was studied by flow cytometry 8 h after infection; enhanced expression of ICAM-1 relative to uninfected cells was observed at 0.1 TCID<sub>50</sub>/cell and peaked at 1 TCID<sub>50</sub>/cell, where there was 3.5-fold induction over uninfected cell levels of ICAM-1 expression (Fig. 1). Based on these dose-response data, a MOI of 1 TCID<sub>50</sub>/cell was utilized in all subsequent studies.

To evaluate the temporal kinetics of ICAM-1 induction by rhinovirus, surface ICAM-1 expression was studied at 0, 1, 4, 8, 16, 24, 48, and 72 h post-rhinovirus 16 infection. Significant up-regulation was apparent within 4 h, was maximal at 8 h, and was still significantly increased at 48 and 72 h after infection (Fig. 2). The levels of rhinovirus-induced ICAM-1 were similar in magnitude to those observed with interferon-γ (10 units/ml) treatment, which was used as a positive control (data not shown). In view of the time course results, an 8-h infection was chosen for comparative studies to investigate the receptor specificity and virus specificity of the up-regulation.

Having investigated rhinovirus regulation of ICAM-1 expression in the human lung carcinoma epithelial cell line A549, we wished to determine whether the same effects could be observed in primary human bronchial epithelial cells. The effect of rhinovirus infection on respiratory epithelial ICAM-1 surface expression was studied by flow cytometry in primary human bronchial epithelial cells. We observed that rhinovirus infection for 8 h increased ICAM-1 expression 12.7 times the control of sham-infected cells (Fig. 3). These data confirmed that rhinovirus infection of both A549 cells and primary bronchial epithelial cells were associated with markedly increased ICAM-1 surface expression. We therefore investigated the mechanisms of this induction in A549 cells, since the numbers of cells required for subsequent experiments precluded the use of primary cells.

The Effects of Rhinovirus Replication and Receptor Binding on Rhinovirus-induced ICAM-1 Cell Surface Expression—Since the virus inoculum was a crude preparation, we wished to confirm that the induction of ICAM-1 surface expression was a result of virus-specific effects rather than a result of stimulation by other soluble products such as cytokines present in the inoculum. We were also interested in investigating whether any rhinovirus-specific effect observed was a result of virus replication or of virus-receptor binding. We therefore elected to inactivate rhinovirus by two methods: UV inactivation to prevent replication but not receptor binding and precoating with soluble receptor (sICAM) to prevent receptor binding. Finally we filtered the inoculum through a molecular weight filter to remove all virus particles and RNA but not small molecules such as cytokines.

As can be observed in Fig. 4, incubation with UV-inactivated virus resulted in marked inhibition of rhinovirus-induced ICAM-1 surface protein expression (from 3.49 ± 0.3 to 1.63 ± 0.1-fold induction); however, there was still a small but significant induction with UV-inactivated rhinovirus over control cells (p < 0.05). In contrast, sICAM inactivated and filtered virus completely abrogated the induction observed with rhinovirus (Fig. 4). These results suggest that approximately one-quarter of the ICAM-1 up-regulation observed with live rhinovirus occurs independently of viral replication, as a result of virus-receptor interaction, while the major part is dependent upon viral replication.

Rhinovirus Induction of ICAM-1 Is Not Virus Receptor/Strain-specific—The major group (90%) of rhinoviruses use members of the low density lipoprotein receptor family to enter cells. Since other members of the receptor family are not members of the low density lipoprotein receptor family, it would appear that rhinovirus is not dependent upon these receptors for entry into the cell. To test this hypothesis, we used a second receptor, the transferrin receptor, which is not a member of the low density lipoprotein receptor family. As can be observed in Fig. 5, we were able to infect A549 cells with rhinovirus using a different receptor, the transferrin receptor, indicating that rhinovirus is not dependent upon these receptors for entry into the cell.
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**FIG. 2.** Time course of rhinovirus-induced ICAM-1 surface expression on A549 respiratory epithelial cells. Surface ICAM-1 expression was measured by flow cytometry on A549 epithelial cells cultured with medium alone (control) or rhinovirus 16 at an MOI of 1 for 1, 4, 8, 16, 24, 48, and 72 h. ICAM-1 induction by rhinovirus infection is expressed as -fold increase over control uninfected cells. Data are mean ± S.E. of at least four separate experiments (*, p < 0.01; **, p < 0.001 compared with control). Rhinovirus infection of A549 respiratory epithelial cells induced a significant up-regulation of ICAM-1 surface expression within 4 h after inoculation, which peaked at 8 h after infection and was still evident up to 72 h.

**FIG. 3.** Rhinovirus-induced ICAM-1 surface expression on primary bronchial epithelial cells. Surface ICAM-1 expression was measured by flow cytometry on primary bronchial epithelial cells cultured with medium alone (control) or rhinovirus 16 at an MOI of 1 for 8 h. ICAM-1 induction by rhinovirus infection is expressed as -fold increase over control uninfected cells. Data are mean ± S.E. of four separate experiments (***, p < 0.001 compared with control). Rhinovirus infection of primary bronchial epithelial cells induced a marked up-regulation of ICAM-1 surface expression 8 h after inoculation.

**FIG. 4.** Effect of inactivation of virus replication and prevention of virus-receptor binding on rhinovirus induction of ICAM-1 surface expression on A549 respiratory epithelial cells and evaluation of serotype and receptor type specificity. Surface ICAM-1 expression was measured by flow cytometry on A549 epithelial cells cultured for 8 h under the following conditions: medium alone (control); live rhinovirus 16 at an MOI of 1 (RV16); UV-inactivated rhinovirus 16 (UV RV16); sICAM-1-pretreated rhinovirus 16 (sICAM RV16); rhinovirus 16 physically removed by filtration (Filtered RV16); live rhinovirus 9 at an MOI of 1 (RV9); live rhinovirus 2 at an MOI of 1 (RV2); and sICAM-1-pretreated rhinovirus 2 (sICAM-1 RV2). ICAM-1 induction by rhinovirus infection is expressed as -fold increase over control uninfected cells. Data are mean ± S.E. of at least four separate experiments (*, p < 0.01; **, p < 0.001 compared with control). Live rhinovirus type 16 (major group), using ICAM-1 as virus receptor induced a greater than 3-fold increase in ICAM-1 surface expression compared with control uninfected cells. Inactivation by physical removal of virus particles (Filtered RV16) and prevention of virus-receptor binding (sICAM RV16) completely abrogated rhinovirus induction of ICAM-1 expression, while prevention of viral replication (UV RV16) only partly inhibited rhinovirus-induced ICAM-1 expression. Other rhinovirus serotypes, rhinovirus 9 (major group) and rhinovirus 2 (minor group), were equally able to up-regulate surface ICAM-1 expression, while pretreatment of rhinovirus 2 (which does not use ICAM-1 as virus receptor) with irrelevant soluble receptor (sICAM-1 RV2) had no effect on rhinovirus induction of ICAM-1 expression.

We therefore compared the stimulatory effect of rhinovirus 16, rhinovirus 9 (both major group), and rhinovirus 2 (minor group), all at an MOI of 1, on A549 cell ICAM-1 surface expression at 8 h postinfection. As shown in Fig. 3, rhinovirus 16, rhinovirus 9, and rhinovirus 2 were equally effective at increasing ICAM-1 surface expression, demonstrating that rhinovirus-induced ICAM-1 up-regulation occurs with at least three of the many different rhinovirus serotypes and that the induction was not receptor-restricted. Furthermore, pretreatment of rhinovirus 2 with sICAM did not alter the ability of this minor group rhinovirus to induce ICAM-1. Having observed that sICAM pretreatment completely abolished (Fig. 4) the ICAM-1 expression induced by the major group rhinovirus, rhinovirus 16, these findings support our interpretations of the preceding data relating to the respective contributions of virus-receptor binding and virus replication to ICAM-1 induction by rhinoviruses.

**Induction of ICAM-1 mRNA in A549 Cells by Live and Inactivated Rhinovirus—**Having found rhinovirus-induced increases in ICAM-1 epithelial cell surface protein expression, we wished to test the effects of rhinovirus infection on epithelial cell ICAM-1 mRNA expression.

First, we wished to determine that the PCR analysis was quantitative over the range of input RNA used in the study. To investigate this, a cell lysate known to produce a strong band upon PCR was serially diluted 3-fold and subjected to extraction, RT-PCR, and densitometric analysis in triplicate. These studies clearly demonstrated that the ICAM-1 RT-PCR used in the subsequent studies was quantitative in a linear fashion (Fig. 5).

The time course of ICAM-1 mRNA induction in response to
rhinovirus 16 was studied by RT-PCR at 0, 1, 3, 6, 8, 12, 16, and 24 h after rhinovirus infection. A549 cells incubated with medium alone did not contain detectable levels of ICAM-1 mRNA (Fig. 6). In accordance with our findings on surface expression, a consistent response to rhinovirus infection was noted, with clear time-dependent increases in ICAM-1 mRNA expression being induced by rhinovirus infection (Table III). A representative experiment is depicted in Fig. 6, where an early increase in levels of ICAM-1 mRNA was detectable at 1 h, peaked at 8 h, and reduced toward but not as far as base line up to 24 h.

Also consistent with the cell surface expression, UV-inactivated virus (Fig. 7, lane 3) resulted in a marked but incomplete inhibition of ICAM-1 mRNA induction compared with live virus (Fig. 7, lane 2), whereas sICAM pretreatment (Fig. 7, lane 4) or filtration (Fig. 7, lane 5) of the virus completely abrogated the response (Table IV).

**Rhinovirus Infection of A549 Cells Up-regulates ICAM-1 Gene Transcription**—To determine whether the observed increases in ICAM-1 mRNA and protein expression in response to rhinovirus infection of A549 cells were mediated by increased ICAM-1 gene transcription, *de novo* synthesis of ICAM-1 mRNA (nuclear run-off) was studied in nuclei obtained from A549 cells after a 1-h rhinovirus infection and in control noninfected cells.

In accordance with the observed mRNA time course studies, ICAM-1 mRNA was undetectable in nuclei from control noninfected cells, either before (Fig. 8, lane 1) or after (Fig. 8, lane 2) *in vitro* transcription, while a weak band of ICAM-1 mRNA was detectable after a 1-h rhinovirus 16 infection but without *in vitro* transcription (Fig. 8, lane 3). The amount of ICAM-1 mRNA was markedly increased by 45-min *in vitro* transcription (Fig. 8, lane 4), indicating that rhinovirus infection of A549 cells resulted in increased *de novo* ICAM-1 mRNA transcription (Table V). This was confirmed by the fact that the rhinovirus-induced increase in ICAM-1 mRNA observed during *in vitro* transcription was abolished in the presence of a-amanitin, a DNA-dependent RNA polymerase II inhibitor (30), (Fig. 8, lane 5; Table V). From these results, we concluded that rhinovirus infection of A549 cells induces a rapid increase in ICAM-1 gene transcription.

**Rhinovirus Infection of A549 Cells Increases ICAM-1 Promoter Activity**—Having demonstrated rhinovirus induction of ICAM-1 gene transcription, we then carried out studies to determine whether rhinovirus infection of A549 cells increased ICAM-1 promoter activity. A549 cells were transiently transfected with constructs containing the CAT reporter gene, whose transcription was regulated by portions of the ICAM-1 promoter. Time course experiments with a construct containing the full-length promoter (−1160 bp) showed that rhinovirus 16-infected cells had significantly increased ICAM-1 promoter activity compared with control cells at all of the tested time points (24, 48, 72, 96, 120 h, data not shown). At 24 h of infection, induction of ICAM-1 promoter activity was maximal, promoter activity being barely detected in control cells (acetylation 2 ± 0.7%), while it was markedly increased in the rhinovirus-infected cells (acetylation 31.9 ± 9%, p < 0.01). The 24-h time point was therefore utilized in subsequent experiments to investigate rhinovirus induction of shorter deletions of the ICAM-1 promoter, to identify the precise sites in the promoter induced by rhinovirus infection.

| Time after rhinovirus infection (h) | ICAM-1 mRNA expression (ICAM-1/APRT densitometric ratio (mean ± S.E.M)) |
|-----------------------------------|--------------------------------------------------|
| 0 (control)                      | 0.27 ± 0.02                                     |
| 1                                 | 0.78 ± 0.09                                     |
| 6                                 | 1.09 ± 0.05                                     |
| 8                                 | 1.34 ± 0.07                                     |
| 12                                | 1.23 ± 0.06                                     |
| 16                                | 0.88 ± 0.06                                     |
| 24                                | 0.55 ± 0.07                                     |

**Table III**

**Time course of rhinovirus induction of ICAM-1 mRNA expression in A549 cells**

**Fig. 5. RT-PCR for ICAM-1 mRNA expression in A549 respiratory epithelial cells is quantitative.** Rhinovirus-infected cell lysate was serially diluted 3-fold and subjected to extraction, RT-PCR, and densitometric analysis. Each point represents the mean ± S.E. optical density of three separate extractions/RT-PCRs. There is a linear relationship between the degree of dilution of input template RNA and the optical density, indicating that the ICAM-1 RT-PCR used in the subsequent studies was quantitative.
Rhinovirus Infection Induces Multiple Transcription Factors

**Rhinovirus Infection Induces ICAM-1 via NF-κB**

**TABLE IV**

| Condition of rhinovirus inoculum | ICAM-1 mRNA expression (ICAM-1/APRT densitometric ratio (mean ± S.E.)) |
|---------------------------------|---------------------------------------------------------------------|
| Control                         | 0                                                                   |
| Rhinovirus 16                    | 1.46 ± 0.08                                                         |
| UV-inactivated rhinovirus 16     | 0.32 ± 0.04                                                         |
| siICAM-inactivated rhinovirus 16 | 0                                                                   |
| Filtered inoculum               | 0                                                                   |

**TABLE V**

| Condition                  | ICAM-1 mRNA expression (ICAM-1/APRT densitometric ratio (mean ± S.E.)) |
|----------------------------|-----------------------------------------------------------------------|
| Control before IVT         | 0                                                                     |
| Control after IVT          | 0                                                                     |
| Rhinovirus 16 before IVT   | 0.11 ± 0.015                                                          |
| Rhinovirus 16 after IVT    | 0.38 ± 0.012                                                          |
| Rhinovirus 16 after IVT with α-amanitin | 0.12 ± 0.013 |

*IVT, in vitro transcription.*

Time course experiments demonstrated that induction of these complexes was maximal 30 min after infection and decreased with longer incubations up to 2 h (Fig. 9A). Competition experiments were then carried out to confirm the specificity of the binding. The addition of excess unlabeled specific (−199/−170) oligonucleotide blocked binding of both of the protein complexes (Fig. 9B, lanes 1 and 2), confirming the specificity of the binding.

Further competition experiments were carried out to identify the transcription factors binding to the probe. The addition of unlabeled consensus NF-κB probe completely blocked the binding of complexes to the specific probe, while the addition of an unlabeled consensus AP-1 probe had no effect (Fig. 9B, lanes 3 and 4), suggesting that both binding complexes were formed of cells that were absent in nuclear extracts from uninfected cells.

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**FIG. 7.** Effect of inactivation of virus replication and prevention of virus-receptor binding upon rhinovirus induction of ICAM-1 mRNA expression in A549 respiratory epithelial cells. Representative (of at least three separate experiments) RT-PCR analysis for ICAM-1 and APRT (housekeeping gene) expression in A549 respiratory epithelial cells uninfected (lane 1) or incubated for 8 h with live rhinovirus 16 (lane 2), UV-inactivated rhinovirus 16 (lane 3), rhinovirus 16 precoated with siICAM-1 (lane 4), or rhinovirus 16 removed by filtration (lane 5) is shown. Above are ethidium bromide-stained gel electrophoreses of products of RT-PCR for APRT and ICAM-1, and below are the ICAM-1/APRT ratios determined by densitometric analysis. Rhinovirus induction of ICAM-1 mRNA expression (lane 2) was completely abolished by prevention of virus-receptor binding by siICAM-1 precoating and by filtration (lanes 4 and 5) and was partly reduced by prevention of viral replication by UV inactivation (lane 3).

**FIG. 8.** Rhinovirus induction of de novo ICAM-1 gene transcription in A549 respiratory epithelial cells. Nuclei from uninfected or rhinovirus-infected A549 cells were used for a nuclear in vitro transcription assay. Representative (of at least three separate experiments) RT-PCR for ICAM-1 and APRT (housekeeping gene) were performed on nuclear RNA obtained from A549 cells incubated in each of the following conditions: uninfected control cells before (lane 1) and after (lane 2) in vitro transcription; rhinovirus 16-injected cells (1 h at an MOI of 1) before (lane 3) and after (lane 4) in vitro transcription; and cells after in vitro transcription in the presence of α-amanitin (lane 5). No ICAM-1 mRNA was detectable in control uninfected cells, either before or after in vitro transcription (lanes 1 and 2, respectively). Increased expression of ICAM-1 mRNA was just detectable after 1-h rhinovirus infection but before in vitro transcription (lane 3). ICAM-1 mRNA expression was markedly increased by 45-min in vitro transcription (lane 4), this increase is completely inhibited by the addition of the RNA polymerase II inhibitor, α-amanitin (lane 5).
Rhinovirus infection of A549 respiratory epithelial cells induces binding of nuclear transcription factors to the −187−178 NF-κB site in the ICAM-1 promoter within 30 min of infection. A, the time course of induction by rhinovirus of nuclear transcription factors
proteins binding to the ICAM-1 kB binding site.

In order to confirm this, competition experiments with −190/−170 probes containing mutated NF-κB and C/EBP sites were then carried out. Binding of these complexes was not affected by competition with the probe containing an intact C/EBP site but a mutated ICAM-1 NF-κB site (M₁), but binding was completely abrogated by competition with the probe containing an intact NF-κB site but a mutated C/EBP site (M₁) (Fig. 9C, lanes 3–5). These results confirmed that rhinovirus infection of A549 cells induces transcription factors binding to the −187/−178 NF-κB cis element but not the −199/−196 C/EBP element in the ICAM-1 promoter.

To determine which members of the NF-κB/Rel protein family were responsible for the formation of the two inducible nuclear complexes, specific antisera directed against members of the NF-κB/Rel family (p50, p52, p65, c-Rel, and Rel-B) were studied. Antiserum specific for p65 clearly supershifted DNA-protein complexes, while the antiserum directed against p50 and c-Rel significantly diminished the formation of the inducible complexes (Fig. 10). In contrast, antibodies to p52, Rel-B, and preimmune serum had no significant effect on complex formation. These studies demonstrated that the most important members of the NF-κB/Rel family mediating rhinovirus-induced NF-κB element binding were p65, c-Rel, and p50, with p65 being the major component of the homo- or heterodimers formed.

−227 to −200 probe Containing an Sp1 Binding Site (−206/−201)—The EMSA resulted in the retardation of two complexes, but no induction was observed in nuclear extracts from rhinovirus-infected cells at any time points up to 2 h (data not shown), indicating that proteins binding to this DNA segment containing an Sp1 binding site are not induced by rhinovirus infection of A549 cells.

−294 to −266 Probe Containing an AP-1 Binding Site (−284/−278)—A single protein-DNA complex was clearly induced in nuclear extracts from rhinovirus-infected A549 epithelial cells compared with noninfected cells, induction being maximal at 30 min and fading thereafter (data not shown). Competition experiments with specific and consensus AP-1 competitor complexes abrogated the signal, while an irrelevant (Sp1) competitor did not, confirming the AP-1 specificity of the signal (data not shown). These data suggest that proteins binding to the AP-1 motif at −284/−278 in the ICAM-1 promoter are also induced in the nuclei of A549 cells by rhinovirus infection. −74 to −43 Probe Containing an NF-κB Binding Motif (−62/−53) Overlapping with an Sp1 Binding Motif (−59/−44)
The −187/−178 NF-κB binding motif, which is already known to play a role in ICAM-1 induction by cytokines (31, 32), is located within this region of the ICAM-1 promoter, and its sequence is truncated by the −182 deletion. Furthermore, the EMSAs clearly demonstrated rhinovirus induction of nuclear proteins binding to this site. Therefore, for further investigations, constructs were used that specifically tested this site.

First, we examined whether DNA fragments containing either the complete binding motif or the −182 deletion of the motif could confer responsiveness to rhinovirus 16 in a heterologous promoter, the herpes simplex minimal thymidine kinase promoter contained in the plasmid pBRAMScat2 (31). Constructs containing the complete NF-κB −187/−178 site (pBRAMScat2 −199/−170 ICAM-1), the truncated site (pBRAMScat2 −199/−182 ICAM-1), and the minimal promoter alone with no ICAM-1 promoter sequence (pBRAMScat2) were transfected in A549 cells. Fig. 12 shows that only when the entire ICAM-1 −187/−178 NF-κB site is present (pBRAMScat2 −199/−170 ICAM-1), is the heterologous thymidine kinase promoter responsive to rhinovirus, confirming that this site is required intact for rhinovirus induction of ICAM-1 promoter activity and that it is also sufficient in the presence of a heterologous minimal promoter.

Finally, to investigate whether the −187/−178 NF-κB site in the ICAM-1 promoter is essential for rhinovirus induction of ICAM-1 promoter activity to occur, constructs containing either the longest ICAM-1 promoter sequence (Fig. 13, −1160 ICAM-1) or the same construct with mutations from TG-GAAATTCC to TctAgATTag at the −187/−178 NF-κB site (−1160 mICAM-1) were used to transfect A549 cells. As shown in Fig. 13, mutation of the −187/−178 NF-κB site completely abrogated rhinovirus induction of ICAM-1 promoter activity, confirming that this NF-κB binding site is necessary for rhinovirus induction of ICAM-1 gene transcription.

**DISCUSSION**

In these studies, we have investigated mechanisms involved in rhinovirus-induced asthma exacerbations by studying the effect of rhinovirus infection on airway epithelial cell ICAM-1 expression. These studies were performed, since ICAM-1 is the receptor for 90% of rhinoviruses and is an adhesion protein that has a central role in inflammatory cell recruitment to the lower airway following rhinovirus infection. ICAM-1 is therefore likely to play a very important role in the mechanisms of virus-induced asthma exacerbations.

We have demonstrated that rhinovirus infection of both primary bronchial epithelial cells and the type II respiratory epithelial cell line A549 markedly increases cell surface expression of ICAM-1. We then investigated the mechanisms of rhinovirus regulation of ICAM-1 expression in A549 cells and observed induction of ICAM-1 promoter activity and increased ICAM-1 mRNA transcription. The rhinovirus induction of ICAM-1 promoter activity was found to involve up-regulation of NF-κB proteins binding to the −187/−178 NF-κB site binding site on the ICAM-1 promoter, and this site was required intact for rhinovirus up-regulation to occur. The principal component of the rhinovirus-induced NF-κB-binding proteins were p65 subunits. These studies elucidate mechanisms probably involved in rhinovirus induction of asthma exacerbations and identify ICAM-1 and NF-κB p65 as potential new targets for development of therapeutic intervention strategies for virus-induced asthma.

Our initial studies demonstrated that rhinovirus infection of A549 respiratory epithelial cells increased the cell surface expression of ICAM-1 protein in a dose-response manner, with peak induction occurring at an MOI of 1 (Fig. 1). The observed increase in ICAM-1 expression peaked at 8 h after virus inoculation.
The intact heterologous minimal promoter.

...inoculation only when the infected over control cells. Data are mean cell lysates was assessed as described under "Materials and Methods." ICAM-1 promoter activation is expressed as fold induction CAT activity in medium alone (control) or rhinovirus 16 at an MOI of 1 for 24 h (RV16). Epithelial cells were harvested, and CAT activity in protein equivalent of the ICAM-1 promoter inserted upstream (pBRAMScat2) by other major group virus particles. A recent report goes some may render cells more, rather than less, susceptible to infection thought to prevent superinfection. Our findings that rhinoviruses induce increased expression of their own receptor receptor- or serotype-restricted. These observations are in keeping with previous observations relating to rhinovirus induction of IL-8 and IL-6, suggesting that the mechanisms involved are likely to have broad applicability across all rhinovirus serotypes. Furthermore, since rhinoviruses are responsible for two-thirds of asthma exacerbations in which a virus is identified, the mechanisms involved are likely to be pertinent to the majority of asthma exacerbations.

The observation that rhinovirus infection of respiratory epithelial cells up-regulated ICAM-1 expression is of great interest, since this molecule is not only important in inflammatory cell recruitment and activation in asthma but is also the receptor for the major group 90% of rhinoviruses. Induction of increased expression of its own cell surface receptor is an unusual property for viruses, since previous observations demonstrate that virus infection down-regulates expression of virus cell surface receptors. For example, measles virus and human immunodeficiency virus both induce down-regulation of their receptors CD46 and CD4, respectively, a process thought to prevent superinfection. Our findings that rhinoviruses induce increased expression of their own receptor suggest that the converse occurs and that rhinovirus infection may render cells more, rather than less, susceptible to infection by other major group virus particles. A recent report goes some way toward confirming this hypothesis, in that rhinovirus infection of primary cultures of human tracheal epithelium was found to increase ICAM-1 mRNA expression and similar magnitude increases in ICAM-1 mRNA expression induced by IL-1β were found to increase susceptibility to rhinovirus infection.

We have previously reported rhinovirus induction of IL-8 protein and mRNA from rhinovirus-infected A549 cells and used sICAM-coated and UV-inactivated virus to investigate the relative contributions of virus-receptor binding and virus replication to the observed up-regulation of IL-8. We reported that UV inactivation reduced by about two-thirds, while pre-coating the virus with soluble receptor completely abrogated, the induction of IL-8. These data suggest that part of the observed up-regulation of IL-8 was related purely to virus-
receptor binding, while the remainder required viral replication (16). In the present studies, we observed the same findings relating to sICAM and UV inactivation to be true for rhinovirus induction of ICAM-1 and have extended them to demonstrate that filtering virus particles but not substances with a molecular mass of <30 kDa (most cytokines) from the inoculum completely abrogated the observed ICAM-1 induction (Fig. 4). We also confirmed the receptor specificity of the sICAM inactivation by demonstrating that precoating a minor group (rhinovirus 2) virus with sICAM had no effect on ICAM-1 upregulation (Fig. 4). These additional data add further weight to our previous hypothesis that part of the signal to up-regulate ICAM-1 or IL-8 protein synthesis occurs consequent to virus-receptor binding, but the major part occurs through processes associated with viral replication.

The ability of rhinovirus infection to up-regulate epithelial cell surface expression of ICAM-1, an important molecule in asthma pathogenesis (23), may have particular importance in the mechanisms of virus-induced asthma exacerbations independent of the effects on rhinovirus replication. We have previously demonstrated that rhinovirus colds induce bronchial mucosal CD3+, CD4+, and CD8+ lymphocyte and eosinophil infiltration, with a more persistent intraepithelial eosinophilia in asthmatic subjects (7). Epithelial expression of ICAM-1 is likely to play an important function in retaining both types of inflammatory leukocyte in the epithelium by binding to its ligands CD18/11a and CD18/11b on lymphocytes and granulocytes, respectively. In addition, binding of ICAM-1 to its integrin ligands on leukocytes may activate these cells and lead to secretion of proinflammatory cytokines and mediators (43–46). Induction of ICAM-1 expression on respiratory epithelial cells is therefore likely to be an important mechanism regulating the bronchial mucosal CD3+, CD4+, and CD8+ lymphocyte and eosinophil infiltration observed in rhinovirus infections. Given the important regulatory role of lymphocytes in promoting airway inflammation in asthma, the induction by rhinoviruses of ICAM-1 is a mechanism that represents an attractive target for development of therapeutic interventions aimed at reducing inflammatory cell recruitment and activation in virus-induced asthma exacerbations.

The observations on the time course and lack of receptor or serotype restriction of ICAM-1 induction by rhinoviruses are in keeping with previous reports of rhinovirus induction of proinflammatory cytokine expression in respiratory epithelial cells (14–17). These similarities suggest that there may be common mechanisms involved in the induction of several proinflammatory proteins by rhinoviruses and that further investigation of the cellular/molecular mechanisms involved might lead to the identification of common pathways suitable for targeting of further future therapeutic intervention strategies. Therefore, having demonstrated that rhinovirus infection of respiratory epithelial cells increased ICAM-1 surface protein expression, we wished to investigate the effects of rhinovirus infection on A549 cell ICAM-1 mRNA expression to elucidate the cellular mechanisms involved in more detail. We observed rhinovirus induction of ICAM-1 mRNA occurring within 1 h of virus inoculation and peaking at 8 h (Fig. 6, Table III). Rhinovirus induction of ICAM-1 mRNA was observed up to 24 h post-virus inoculation (no studies were carried out beyond this time point). As we had observed with ICAM-1 protein expression, UV inactivation partially abrogated the rhinovirus-induced ICAM-1 mRNA expression while inactivating the virus by filtration or by precoating with soluble receptor completely abrogated the signal (Fig. 7, Table IV). These studies confirmed that, as with ICAM-1 protein expression, rhinovirus induction of ICAM-1 mRNA was also consequent partly upon virus-receptor binding and was partly related to viral replication.

Having observed rhinovirus-induction of both ICAM-1 protein and mRNA expression, we hypothesized that rhinovirus infection of A549 cells increased ICAM-1 expression by up-regulating ICAM-1 gene transcription. To investigate this possibility, we analyzed in vitro transcription of ICAM-1 mRNA in rhinovirus-infected and noninfected cells. As seen in Fig. 8 and Table V, we observed clear induction of ICAM-1 gene transcription by rhinovirus infection and inhibition of this induction by an inhibitor of RNA polymerase II, α-amanitin. These data confirmed that rhinovirus infection of A549 respiratory epithelial cells rapidly increased de novo transcription of ICAM-1 mRNA.

Next, we wished to determine the molecular mechanisms involved in rhinovirus induction of ICAM-1 mRNA transcription, since these mechanisms might identify a target for development of new therapeutic intervention strategies. We focused on transcription factor-mediated activation of the ICAM-1 promoter, since previous observations indicated that both IL-6 induction by rhinoviruses (15) and IL-8 induction by respiratory syncytial virus (47) were dependent on proteins binding to NF-κB sites in the relevant promoters, and our own observations had indicated that the presence of both an NF-κB site and an AP-1 site was required for induction of the IL-8 promoter by rhinovirus infection (48).

The ICAM-1 promoter contains several potential transcription factor binding sites, several of which have been implicated in the induction of ICAM-1 gene transcription in response to various proinflammatory stimuli, such as PMA and cytokines (31, 32). In the present study, we observed that rhinovirus infection of A549 epithelial cells induced proteins binding to
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the both the –187/–178 and the –62/–53 ICAM-1 NF-κB sites (Fig. 9 and data not shown) and the –284/–278 AP-1 site (data not shown) but neither the –206/–201 nor –59/–54 Sp1 site. We therefore performed reporter gene assays to determine which of these sites was functional in rhinovirus induction of ICAM-1 promoter activity. We observed that sequential deletion of the ICAM-1 promoter up to –277 base pairs from the transcription initiation site had no effect on rhinovirus induction of ICAM-1 promoter activity (Fig. 11). These data suggest that despite the fact that proteins binding to this site are induced by rhinovirus infection of A549 cells, the –284/–278 AP-1 site is not required for rhinovirus induction of ICAM-1 promoter activity to occur. In contrast, deletion of the promoter to –182 bases resulted in complete loss of rhinovirus inducibility, suggesting that elements contained within the –182 to –277 region were necessary for, and that the –62/–53 NF-κB site alone was insufficient for, rhinovirus-induced up-regulation of ICAM-1 promoter activity to occur (Fig. 11).

The –182 deletion interrupts the –187/–178 NF-κB site. Given the previously observed functionality of this site in ICAM-1 induction by cytokines (32), we hypothesized that this site might also be important in rhinovirus induction of ICAM-1 promoter activity. Mutational analysis was therefore carried out, with reporter gene assays being performed with full-length ICAM-1 promoter and with full-length ICAM-1 promoter with the –187/–178 NF-κB site mutated in four nucleotide positions. Despite the presence of the full-length promoter, mutation of this –187/–178 NF-κB site completely abrogated rhinovirus induction of ICAM-1 promoter activity (Fig. 13), confirming that this site was required intact for rhinovirus induction of ICAM-1 promoter activity to occur. The importance of this –187/–178 NF-κB site in rhinovirus induction of promoter activity was then investigated using plasmids containing the reporter gene linked to the thymidine kinase minimal promoter alone and with the truncated and intact versions of the –187/–178 NF-κB site (Fig. 12). These data confirmed that this site was required intact for rhinovirus induction of a heterologous promoter to occur and that it was sufficient in the presence of a basic minimal promoter.

Previous studies have demonstrated that members of the NF-κB family of transcription factors are important in induction of proinflammatory cytokines by both rhinovirus and respiratory syncytial virus (15, 47, 48). These data suggest that NF-κB may play a very important role in the induction of proinflammatory cytokines by virus infections in general, and our data reported herein extend these observations to include proinflammatory adhesion molecules. The NF-κB/Rel family of transcription factors contains several members, so far including p50, p52, p65, c-Rel, and Rel-B, which are capable of forming homo- or heterodimers. We performed supershift experiments to investigate which members of the NF-κB/Rel family were induced by rhinovirus infection and demonstrated that the major component of rhinovirus-induced proteins binding to the –187/–178 NF-κB site in the ICAM-1 promoter were p65 proteins, with smaller amounts of p50 and c-Rel (Fig. 10). These data support previous observations that the major rhinovirus-induced proteins binding to the IL-6 promoter in A549 cells are also p65 homodimers or heterodimers (15).

In addition to the early important study in primates by Wegner et al. (23), two further recent studies have reported important roles for ICAM-1 in promoting inflammation in asthma by demonstrating its important role in promoting lymphocyte and eosinophil infiltration in murine models (24, 25). Given the marked bronchial lymphocyte and eosinophil infiltration observed in virus-induced asthma (7), its role as the rhinovirus receptor, and our demonstration that rhinovirus infection induces increased ICAM-1 expression in respiratory epithelial cells, we believe that ICAM-1 is likely to play a critical role in promoting lower airway inflammation in virus-induced asthma. This hypothesis is supported by the fact that we and others (49) have observed increased sICAM levels in nasal secretions during rhinovirus infections. Recent support for the role of ICAM-1 up-regulation in rhinovirus-induced asthma also comes from the demonstration that experimental rhinovirus infection of asthmatic subjects up-regulates ICAM-1 expression on bronchial epithelial cells in vivo (50). These data make epithelial ICAM-1 a prime target for therapeutic intervention strategies in virus-induced asthma exacerbations. The fact that anti-ICAM-1 monoclonal antibodies were able to reduce allergen-induced airway hyperreactivity and eosinophil influx in a primate model of asthma (23) also strongly supports the potential of ICAM-1-targeted intervention to ameliorate virus-induced asthma exacerbations.

We have also investigated the mechanisms of rhinovirus induction of ICAM-1 and have observed an important role for NF-κB-mediated transcriptional up-regulation, the major component of which is contributed by p65. Given that transcriptional up-regulation via NF-κB is also necessary for induction of IL-6 (15) and IL-8 (48) by rhinoviruses and that in the case of IL-6 p65 is again the major component, this molecule also represents an important potential target for future therapeutic intervention in virus-induced asthma exacerbations.

In conclusion, we have demonstrated that rhinovirus infection of respiratory epithelial cells increases surface ICAM-1 expression via NF-κB p65-mediated transcriptional up-regulation. We believe that these two molecules (ICAM-1 and NF-κB p65) represent new targets for potential therapeutic intervention in virus-induced asthma exacerbations.

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