Respiratory Epithelial Cell Expression of Vascular Cell Adhesion Molecule-1 and Its Up-regulation by Rhinovirus Infection via NF-κB and GATA Transcription Factors*

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Vascular cell adhesion molecule 1 (VCAM-1) is an inducible cell surface glycoprotein belonging to the immunoglobulin supergene family. It was first identified as an adhesion molecule induced on endothelial cells by the inflammatory cytokines interleukin (IL)-1 and tumor necrosis factor (TNF) or lipopolysaccharide (1, 2). VCAM-1 plays a central role in inflammatory cell recruitment and accumulation at sites of inflammation by binding to its ligand the leukocyte α4β1 integrin VLA-4 on T and B lymphocytes, eosinophils, monocytes, and basophils but not on neutrophils that lack VLA-4 (3, 4). In addition to its importance in inflammatory cell recruitment, VCAM-1 is also involved in both T lymphocyte and eosinophil activation by providing T cell receptor-engaged CD4+ T cells the costimulation required for T cell proliferation, IL-2 receptor expression, and cytokine release (5, 6), and by interaction with its ligand VLA 4 on eosinophils promoting superoxide generation and degranulation (7, 8). Finally, endothelial expression of VCAM-1 is increased in asthma and rhinitis, which are allergic airway conditions characterized by inflammation with lymphocyte and eosinophil infiltration (9–12). These data suggest that VCAM-1 is a crucial molecule in inflammatory cell recruitment, accumulation, and activation at sites of allergic inflammation.

Respiratory virus infections have recently been associated with the majority of asthma exacerbations in both adults and children (13–16). In all these studies, rhinoviruses were the most frequently identified virus type. Rhinovirus-induced asthma exacerbations therefore cause enormous morbidity and represent a major health and economic problem. A better understanding of the mechanisms involved in rhinovirus-induced asthma exacerbations would greatly aid the development of new therapies for this common condition, because to date, no safe effective therapy is available (17, 18).

The mechanisms by which rhinoviruses trigger asthma exacerbations are poorly understood. The lower airway cellular response to experimental RV colds has been recently studied in normal, allergic rhinitic, and asthmatic subjects. Increased numbers of T lymphocytes (19) and eosinophils (19–21) have been reported, and persistent eosinophilia was observed 4–8 weeks after the infection only in allergic rhinitic or asthmatic patients (19, 21). These data, combined with the increased bronchial hyperresponsiveness demonstrated in experimental rhinovirus infections in asthmatic (21, 22) and atopic subjects (23) and the fact that asthma exacerbations have been induced by experimental rhinovirus infections (21, 24), provide strong evidence that rhinovirus-induced bronchial intraepithelial lymphocyte and eosinophil infiltration and activation are likely very important mechanisms in virus-induced asthma exacerbations.

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 (25). Indeed rhinoviruses are capable of prolonged, noncytolytic infection of lower respiratory epithelial cells and induce production of pro-inflammatory cytokines such as IL-6, IL-8, and granulocyte macrophage colony-stimulating factor (26–29).

Endothelial expression of VCAM-1 is important in inflammatory cell recruitment to sites of inflammation, and this can be inhibited by monoclonal antibody to its ligand, VLA-4 (30). However, such treatment involves intravenous delivery of het-
erologous or humanized proteins and as such is expensive and impractical. Basal and inducible epithelial VCAM-1 expression has recently been observed in small bowel, glomerular, and tubular epithelial cells (31–35). The presence of VCAM-1 has been described on glomerular epithelial cells in normal glomeruli in renal biopsies (34–36), and VCAM-1 up-regulation in epithelial cells has been documented in immune-mediated renal disease (33, 37, 38), suggesting that renal epithelial VCAM-1 expression is important in immune-mediated renal diseases.

Two previous experimental studies have failed to demonstrate constitutive or inducible VCAM-1 on bronchial epithelial cells (39, 40), suggesting that respiratory epithelial cells do not express VCAM-1. However, epithelial VCAM-1 is thought to mediate adhesion and penetration of pro-inflammatory leukocytes in tonsilar epithelium (41), inducible expression of VCAM-1 was recently reported in the BEAS-2B bronchial epithelial cell line and soluble VCAM-1 was detected in supernatants of primary bronchial epithelial cells (42), suggesting that respiratory epithelial cells may express VCAM-1 under certain circumstances.

We investigate respiratory epithelial VCAM-1 expression and its modulation by rhinovirus infection and pro-inflammatory stimuli. Having found that rhinovirus infection, but not other pro-inflammatory stimuli, induced VCAM-1 up-regulation in various human respiratory epithelial cell types, we investigated the intracellular mechanisms of rhinovirus induction of VCAM-1 expression to identify potential targets for modulation of rhinovirus-induced VCAM-1 in the therapy of rhinovirus-induced asthma exacerbations.

MATERIALS AND METHODS

Cell Culture—Ohio HeLa cells were obtained from the Medical Research Council Common Cold Unit (Salisbury, UK), and A549 cells, a type II respiratory cell line, were obtained from the American Type Culture Collection (Manassas, VA). 16HBE cells, a differentiated SV-40 transformed bronchial epithelial cell line (43), were a generous gift from Dr. D.C. Gruenert (University of California, San Francisco, CA). Primary human bronchial epithelial cells (HBEc) were obtained by bronchial brushing from normal subjects and cultured as described previously (44). These cells are >95% cytokeratin 18 immunoreactive respiratory epithelial cells as assessed by immunofluorescence microscopy.

Viral Stocks—Rhinoviruses type 16, 9 (major group), and 2 (minor group) were obtained from the Medical Research Council Common Cold Unit. Viral stocks were prepared and titrated by infection of sensitive cell monolayers (HeLa) as described previously (44). Tissue culture infective dose 50% (TCID50) viral values were determined (45), and virus at a multiplicity of infection (MOI) of 1 was used for all the experiments, except where indicated.

Rhinovirus Inactivation—For selected experiments, inactivated rhinovirus type 16 was used, as described previously (28, 44). Inactivation/exclusion of the virus was achieved by (i) precoating the virus with its receptor (ICAM-1), (ii) UV light, or (iii) rhinovirus removal from inocula, by ultrafiltration through 30-kDa cut-off membranes (Amikon, London, UK). For each method, confirmation of inactivation was carried out by microtiter plate assay as described above.

Measurement of VCAM-1 Surface Protein Expression—Flow cytometry was used to quantify the level of expression of VCAM-1 on the surface of resting and stimulated respiratory epithelial cells. 1 × 10^6 A549 or 16HBE cells (24-well plates). Control medium or one of the following stimuli was added when cells were confluent: rhinovirus type 16 at MOI of 1; phorbol 12-myristate 13-acetate (20 ng/ml); lipopolysaccharide (100 mg/ml); IFN-γ (100 units/ml); TNFα (200 units/ml); IFN-γ plus TNFα; IFN-γ plus TNFα and IL-1α (10 units/ml). Incubation continued for various time points between 1 and 72 h. Dose response studies were carried out by using 0.1, 0.2, 0.5, 1, and 2 MOI at 24 h. Similarly, the effect of inactivated virus was studied at 8 h. HBEC (2 × 10^6) were grown in 12-well plates and infected when confluent for 8 h.

At desired time points, cells were harvested, incubated with fluorescein isothiocyanate-conjugated anti-human VCAM-1 (CD 106) antibody or isotype-specific control antibody (Southern Biotechnology Associates, Birmingham, AL), and analyzed for fluorescence by single color flow cytometry as described previously (44). Mean fluorescence intensity was measured and normalized as percentage of noninfected control values, after subtraction of background staining.

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FIG. 1. VCAM-1 surface protein expression on 16HBE epithelial cells. Representative flow cytometric analysis of VCAM-1-specific immunofluorescence with anti CD106 (VCAM-1) monoclonal antibody (dotted line) or isotype matched control monoclonal antibody (solid line) in 16HBE epithelial cells. Cells were in resting (control) conditions. The y axis indicates the number of counted cells/each fluorescence intensity channel, and the x axis shows the associated fluorescence intensity.
Oligonucleotide Probes (Table I)—Double-stranded oligonucleotides containing wild type and mutated sequences of the VCAM-1 promoter NF-κB- and GATA-binding sequences were obtained commercially (Oswell DNA Service, Southampton, UK). For control experiments double-stranded oligonucleotides containing wild type sequences of the ICAM-1 promoter SP-1-binding sequence were used (Oswell DNA Service). Mutant sequences were identical to those used in the mutant reporter constructs. NF-κB, AP-1, and SP-1 consensus double-stranded oligonucleotides were obtained commercially (Promega).

Oligonucleotides were labeled and incubated with 5 μg of nuclear protein as previously reported (44). Complexes were resolved on 5% nondenaturing polyacrylamide gels. Dried gels were autoradiographed at −70°C overnight.

Statistical Analysis—Data were expressed as means ± S.E., and comparison between groups was performed by analysis of variance for multiple comparisons, and by paired Student’s t test for individual comparisons. All experiments were carried out at least three times.

RESULTS

Respiratory Epithelial Cells Constitutively Expresses VCAM-1 Surface Protein—VCAM-1 was constitutively present on both respiratory epithelial cell lines and primary bronchial epithelial cells, with mean fluorescence intensities of 30.2 ± 8.4, 26.3 ± 7.1, and 22.6 ± 4.5 for 16HBE, A549, and HBEC, respectively, after subtraction of background staining. A representative example of 16HBE basal VCAM-1 expression is shown in Fig. 1. Consistent with these findings, VCAM-1 mRNA was clearly detectable in the respiratory epithelial cells under basal conditions when the PCR cycle number was increased to 30, in both first round and nested amplifications (data not shown).

Rhinovirus Infection, but No Other Stimuli Tested, Induces VCAM-1 Cell Surface Protein Expression in Respiratory Epithelial Cells—VCAM-1 expression on 16HBE and A549 cells was measured before and after incubation with phorbol 12-myristate 13-acetate, lipopolysaccharide, IFN-γ, TNFα, IFN-γ and TNFα combined, and the combination of IFN-γ, TNFα, and IL-1β for 8 and 24 h. None of these potent proinflammatory stimuli either alone or in combination were found to up-regulate VCAM-1 expression on 16HBE cells (data not shown).

In contrast to the other stimuli tested, rhinovirus infection induced a significant up-regulation of VCAM-1 expression on both 16HBE and A549 epithelial cells. Dose response studies were performed in A549 and 16HBE cells infected with rhinovirus 16 to determine whether the induction of VCAM-1 oc-
Rhinovirus induction of VCAM-1 expression in both cell types. Other rhinovirus serotypes, rhinovirus 9 (RV9), major group (ICAM-1 as virus receptor) with soluble major group receptor (sICAM) and rhinovirus 2 (minor group) were equally able to up-regulate surface VCAM-1 expression, whereas pretreatment of rhinovirus 2 (which does not use ICAM-1 as virus receptor) with sICAM did not alter the ability of this minor group rhinovirus to induce VCAM-1 (Fig. 3). These findings demonstrate the specificity of rhinovirus inactivation via sICAM-1 binding for the major rhinovirus group.

**Rhinovirus Induction of VCAM-1 Is Not Virus Receptor/Strain-specific**—The major group (90%) of rhinoviruses use ICAM-1 as their cell surface receptor (50, 51), whereas the remainder (minor group) use a member of the LDL-receptor family (52). To investigate whether rhinovirus induction of VCAM-1 up-regulation is strain or receptor restricted, the stimulatory effects of rhinovirus 16, rhinovirus 9 (both major group), and rhinovirus 2 (minor group) were respectively studied on 16HBE (8 h) and in A549 (24 h) epithelial cells. As shown in Fig. 3, rhinovirus 16 and rhinovirus 9, and rhinovirus 2 were equally effective at increasing VCAM-1 surface expression, demonstrating that rhinovirus induction of VCAM-1 occurs with at least three of the many different rhinovirus serotypes and that no strain or receptor specificity is observed. Furthermore, pretreatment of rhinovirus 2 with sICAM did not alter the ability of this minor group rhinovirus to induce VCAM-1 (Fig. 3). These findings demonstrate the specificity of rhinovirus inactivation via sICAM-1 binding for the major rhinovirus group.

**Induction of VCAM-1 mRNA in A549 Cells by Live and Inactivated Rhinovirus**—To determine whether the observed VCAM-1 surface protein up-regulation induced by rhinovirus was accompanied by increased VCAM-1 mRNA expression, the expression of VCAM-1 mRNA in response to rhinovirus 16 was examined in a dose response manner. Cell surface VCAM-1 expression was examined 8 h after infection on the basis of preliminary studies. Enhanced expression of VCAM-1 relative to uninfected cells was observed in both cell lines, starting from 0.5 TCID50/cell and being maximal at 1–2 TCID50/cell (Fig. 2, upper panel). Based on these dose response data, a MOI of 1 was utilized in all subsequent studies.

To evaluate the temporal kinetics of VCAM-1 induction by rhinovirus infection in both cell lines, surface VCAM-1 expression was studied at 0, 1, 4, 8, 16, 24, 48, and 72 h post rhinovirus 16 infection. Similar results were observed in A549 and 16HBE cells, with a significant increase within 4 h, a maximal effect between 8–24 h, and a still detectable up-regulation at 72 h (Fig. 3, lower panel). In view of the time course results, 8 and 24 h infection were respectively chosen for A549 and 16HBE cells to investigate the receptor specificity and virus specificity of the up-regulation.

To investigate whether the findings in cells lines were applicable to primary bronchial epithelium, similar studies were performed with HBEC. A 50% up-regulation of VCAM-1 surface expression was observed on HBEC cells in response to 8 h of rhinovirus 16 infection at a MOI of 1 (data not shown).

The Effect of Rhinovirus Inactivation on Rhinovirus-induced VCAM-1 Cell Surface Expression—Because the virus inoculum was a crude preparation, experiments were carried out to confirm that the induction of VCAM-1 surface expression was the result of virus-specific effects. Inactivation by UV pretreatment and precoating with sICAM and filtration of the virus from the inoculum all completely abrogated the induction of VCAM-1 expression observed with live rhinovirus (Fig. 3) at peak time points in both 16HBE (8 h) and A549 (24 h) cells. These experiments confirmed that the induction of VCAM-1 expression by the inoculum was related to the presence and replication of live rhinovirus.
The time course of VCAM-1 mRNA was studied by RT-PCR at 0, 1, 3, 6, 8, 12, and 24 h after rhinovirus infection. At the cycle numbers used for these experiments, A549 cells incubated with medium alone did not contain detectable levels of VCAM-1 mRNA. In accordance with the findings on surface expression, a consistent response to rhinovirus infection was noted, with an early significant increase in levels of VCAM-1 mRNA, which was detectable at 1 h and that peaked at 8 h (control samples, 0; rhinovirus infection, 1.41 \( \pm \) 0.07 arbitrary units; \( p \), 0.001). A representative time course experiment is depicted in Fig. 4A. Induction of VCAM-1 mRNA expression in response to rhinovirus infection was still present, although it was reduced in comparison with earlier time points, at 24 h (Fig. 4A, lane 4, rhinovirus infection 0.7 \( \pm \) 0.1 arbitrary units, \( p \), 0.001 versus control samples). Uniformity of loaded and processable RNA was assessed by standard housekeeping gene (APRT) RT-PCR. Similar experiments were also carried out in 16HBE cells; rhinovirus induction of VCAM-1 mRNA was also observed in 16 HBE bronchial epithelial cells (data not shown). Because of the large number of cells required for the subsequent studies, all further studies were carried out in A549 cells.

Consistent with the cell surface expression, sICAM pretreatment (Fig. 4B, lane 2), UV inactivation (Fig. 4B, lane 3), and exclusion of the virus by filtration (Fig. 4B, lane 4) completely infection was examined. The time course of VCAM-1 mRNA was studied by RT-PCR at 0, 1, 3, 6, 8, 12, and 24 h after rhinovirus infection. At the cycle numbers used for these experiments, A549 cells incubated with medium alone did not contain detectable levels of VCAM-1 mRNA. In accordance with the findings on surface expression, a consistent response to rhinovirus infection was noted, with an early significant increase in levels of VCAM-1 mRNA, which was detectable at 1 h and that peaked at 8 h (control samples, 0; rhinovirus infection, 1.41 \( \pm \) 0.07 arbitrary units; \( p \), 0.001). A representative time course experiment is depicted in Fig. 4A. Induction of VCAM-1 mRNA expression in response to rhinovirus infection was still present, although it was reduced in comparison with earlier time points, at 24 h (Fig. 4A, lane 4, rhinovirus infection 0.7 \( \pm \) 0.1 arbitrary units, \( p \), 0.001 versus control samples). Uniformity of loaded and processable RNA was assessed by standard housekeeping gene (APRT) RT-PCR. Similar experiments were also carried out in 16HBE cells; rhinovirus induction of VCAM-1 mRNA was also observed in 16 HBE bronchial epithelial cells (data not shown). Because of the large number of cells required for the subsequent studies, all further studies were carried out in A549 cells.

Consistent with the cell surface expression, sICAM pretreatment (Fig. 4B, lane 2), UV inactivation (Fig. 4B, lane 3), and exclusion of the virus by filtration (Fig. 4B, lane 4) completely
suppressed rhinovirus induced VCAM-1 mRNA expression (control samples and all inactivated samples, 0; rhinovirus infected, 1.7 ± 0.08 arbitrary units; p < 0.001). These data demonstrate that rhinovirus-induced VCAM-1 up-regulation is associated with VCAM-1 mRNA accumulation and therefore is regulated at a pretranslational level.

**Rhinovirus Infection of A549 Cells Up-regulates VCAM Gene Transcription**—To investigate the pretranslational mechanisms of increased VCAM-1 expression in response to rhinovirus infection, VCAM-1 gene transcription was studied by a previously reported in vitro transcription assay (44). De novo synthesis of VCAM-mRNA was evaluated in nuclei obtained from A549 cells after 1 h of rhinovirus infection and from control noninfected cells. Rhinovirus infection significantly increased VCAM-1 mRNA transcription (p < 0.001).

In accordance with the observed mRNA time course studies, VCAM-1 mRNA was undetectable in nuclei from control noninfected cells, either before (Fig. 5, lane 1) or after (Fig. 5, lane 2) in vitro transcription, whereas a weak band of VCAM-1 mRNA was detectable after 1 h of rhinovirus 16 infection before in vitro transcription (0.14 ± 0.01 arbitrary units; Fig. 6, lane 3). VCAM-1 mRNA levels were clearly increased by 45 min in vitro transcription (0.3 ± 0.02 arbitrary units; p > 0.01 versus control and before in vitro transcription samples; Fig. 5, lane 4), indicating that rhinovirus infection of A549 cells resulted in increased de novo VCAM-1 mRNA transcription. This was confirmed by the fact that the rhinovirus-induced increase in VCAM-1 mRNA observed during in vitro transcription was abolished in the presence of a-amanitin (0.12 ± 0.01 arbitrary units; p < 0.01 versus after in vitro transcription samples; Fig. 5, lane 5), a DNA-dependent RNA polymerase II inhibitor (53).

From these results we concluded that rhinovirus infection of A549 cells induces a rapid increase in VCAM-1 gene transcription.

**Rhinovirus Infection of A549 Cells Increases VCAM-1 Promoter Activity**—Having demonstrated that rhinovirus infection of respiratory epithelial cells induced VCAM-1 gene transcription, studies were carried out to further investigate the intracellular mechanisms of rhinovirus induction of VCAM-1 gene transcription. Experiments were performed with a CAT construct containing a deletion of the VCAM-1 promoter (−258 bp), which was found most active in other studies of VCAM-1 promoter activity (47). A549 cells infected with rhinovirus 16 for 24 h had markedly increased VCAM-1 promoter activity compared with control cells, promoter activity being barely detected in control cells (acytation, 4.8 ± 2.7%), whereas it was significantly increased in the rhinovirus infected cells (acytation, 31.7 ± 6.5%; p < 0.01).

**Identification of Rhinovirus Response Regions in the VCAM-1 Promoter**—To map the VCAM-1 promoter regions relevant for rhinovirus-induced VCAM-1 gene transcription, A549 cells were transiently transfected with constructs containing CAT reporter genes whose transcription was regulated by sequential deletions (Fig. 6A) of the VCAM-1 promoter, and the effect of rhinovirus infection on CAT activity was studied.

As seen in Fig. 6B, promoter activity of the CAT constructs under the control of the proximal −755, −518, and −258 bp of the VCAM-1 promoter was strongly induced by rhinovirus 16 infection of A549 cells. This inducibility was reduced by 50–60% with the construct containing the proximal −98 bp of the VCAM-1 promoter. Further deletions of the VCAM-1 promoter completely abolished the capacity of rhinovirus infection to induce VCAM-1 promoter activity. These studies indicated the presence of DNA sequences necessary for rhinovirus induction of VCAM-1 promoter activity between the positions −258/−98 and −98/−44 relative to the transcription initiation site.

**Rhinovirus Infection Induces Binding of NF-κB and GATA Transcription Factors to the VCAM-1 Promoter**—Sequence analysis of the proximal VCAM-1 promoter has revealed potential binding sites for several transcription factors including two GATA elements in the region −258/−98 (−254/−251 and −239/−236), and two consensus NF-κB elements in the region −98/−44 (−72/−63 and −52/−48) (Fig. 6A). Nuclei were extracted from infected and uninfected A549 respiratory epithelial cells, lysed, and analyzed by electrophoretic mobility shift
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**Fig. 7.** Rhinovirus infection of A549 respiratory epithelial cells induces binding of nuclear transcription factors to the GATA sites in the −258/-98 bp region of the VCAM-1 promoter. 

A, the time course of induction by rhinovirus of nuclear transcription factors binding to the −258/−232 (containing two GATA sites, Table I) portion of the VCAM-1 promoter was assessed by EMSA. Nuclear extracts were prepared from uninfected (time 0) and rhinovirus 16 infected A549 cells at various time points (30, 60, and 90 min) after infection and incubated with radiolabeled −258/−232 VCAM-1 (Table I) probe. Resolution of binding complexes was accomplished on 5% polyacrylamide gels. Representative radiograph of one of at least three separate experiments. Two retarded complexes binding to the −258/−232 portion of the VCAM-1 promoter were induced in nuclei from rhinovirus infected cells, with peak induction of binding activity being observed within 30 min of rhinovirus infection. Induction of binding activity by rhinovirus reduced gradually from 30 up to 90 min. B, the specificity of the binding activity induced by rhinovirus infection of A549 cells was examined by EMSA with competition studies. Nuclear extracts from A549 cells uninfected (lane 7) or rhinovirus 16-infected for 30 min (lanes 1–6) were studied. Radiolabeled −258/−232 VCAM-1 probe was used in the absence (lanes 2 and 7) or in the presence of excess unlabeled specific −258/−232 probe (SC, lane 3), excess unlabeled specific −258/−232 probe mutated at the GATA sites (mGATA, Table I) (lane 4), and excess unlabeled probes containing consensus AP-1-binding sequences (lane 5). Radiolabeled mGATA probe (Table I) was used in lane 6. Resolution of binding complexes was accomplished on 5% polyacrylamide gels. Representative radiograph of one of at least three separate experiments. Specificity of the binding activity induced by rhinovirus infection of A549 cells to the −258/−232 portion of the VCAM-1 promoter was confirmed by complete inhibition of the induction of binding activity with excess unlabeled specific probe (lane 3). Involvement of the GATA sites in this binding activity was confirmed by the lack of competition with excess unlabeled specific −258/−232 probe mutated at the GATA sites (mGATA, lane 4) or irrelevant probe (consensus AP-1, lane 5). This result was also confirmed by complete assays (EMSA) using labeled probes containing each of the potential binding sites in the VCAM-1 promoter.

−258 to −232 Probe Containing Two (−254/−251 and −239/−236) GATA Sites—Two retarded complexes were observed using nuclear extracts from rhinovirus 16 infected A549 cells that were faintly detectable in nuclear extracts from uninfected cells. Time course experiments showed that binding of these complexes was maximal 30 min after rhinovirus infection and decreased with longer incubations up to 90 min (Fig. 7A). Competition experiments were then carried out to confirm the specificity of the binding. Addition of excess unlabeled specific (−258/−232) oligonucleotide blocked the induction of complexes (Fig. 7B, lanes 2 and 3), confirming the specificity of the binding activity.

Further competition experiments were carried out to identify the transcription factors binding to the probes. Formation of these complexes was not affected by competition with excess heterologous oligonucleotide (consensus AP-1) (Fig. 7B, lane 5) or with excess unlabeled DNA oligonucleotide containing mutated sequences of both VCAM-1 promoter GATA-binding sites (mGATA; Table I and Fig. 7B, lane 4), indicating that binding complexes were formed of proteins binding specifically to the VCAM-1 promoter GATA-binding sites.

To confirm this, the same mGATA oligonucleotide was radiolabeled and used directly as probe. DNA-protein complex formation was not observed with this labeled probe (Fig. 7B, lane 6), again confirming that rhinovirus infection of A549 cells induces transcription factors binding specifically to the −254/−251 and −239/−236 GATA elements of the VCAM-1 promoter.

−64 to −45 Probe Containing an NF-κB-binding Site (−571/−48)—Because both NF-κB sites contained in the VCAM-1 promoter are consensus sites, we investigated induction by rhinovirus of proteins binding to only one of these sites, the −571/−48 site. Two protein-DNA complexes were clearly induced in nuclear extracts from rhinovirus-infected A549 cells compared with noninfected cells. This effect again showed a rapid kinetic peak at 30 min after infection and fading thereafter (Fig. 8A). Competition experiments with excess specific and consensus NF-κB competitor unlabeled probes completely abrogated the signal (Fig. 8B, lanes 2 and 3), whereas an irrelevant (AP-1) competitor did not (Fig. 8B, lane 4), confirming the NF-κB specificity of the binding. These data indicate that proteins binding to the NF-κB sites in the VCAM-1 promoter are also induced in the nuclei of A549 cells during rhinovirus infection.

**Lack of Rhinovirus Induction of Proteins Binding to the ICAM-1 Promoter −227 to −200 DNA Segment Containing an SP1-binding Site (−206/−201)—**To confirm that the changes observed in binding to GATA and NF-κB were direct specific effects of rhinovirus infection and not nonspecific modifications possibly because of variations in nuclear extract integrity or in complex binding competence, we tested by EMSAs the same nuclear extracts using a radiolabeled probe consisting of −227 to −200 of the ICAM-1 promoter containing an SP1-binding site. Proteins binding to this probe have previously been shown not to be modified by rhinovirus infection (44).

The EMSA resulted in the retardation of two complexes, but no induction was observed after rhinovirus infection up to 2 h (Fig. 9). Competition experiments confirmed the SP1 specificity of the binding (data not shown). These data indicate that protein binding to this DNA segment is specific to the SP1-binding
site and that no induction of protein binding occurred during rhinovirus infection and thus confirm the specificity of the induction of proteins binding to the VCAM-1 NF-κB- and GATA-binding sites reported above. Having observed rhinovirus-specific induction of proteins capable of binding both NF-κB- and GATA-binding sites within the VCAM-1 promoter, we then carried out reporter gene assays to determine whether the potential candidate transcription factor-binding sites were functional in rhinovirus induction of VCAM-1 promoter activity.

**DISCUSSION**

We have investigated mechanisms involved in rhinovirus-induced asthma exacerbations by studying the effect of rhinovirus infection on airway epithelial cell VCAM-1 expression. These studies were performed as intraepithelial lymphocyte and eosinophil recruitment in the lower airway is an important feature of rhinovirus-induced asthma exacerbations, and VCAM-1 is an adhesion protein that has a central role in recruitment and activation of these cell types. We hypothesized that respiratory epithelial VCAM-1 expression is likely to play an important role in the mechanisms of virus-induced asthma exacerbations. We therefore investigated respiratory epithelial cell VCAM-1 expression and regulation by rhinovirus infection and several pro-inflammatory stimuli to identify new targets for treatment of virus-induced asthma exacerbations.

Our initial studies demonstrated that each of A549 pulmonary and 16 HBE bronchial cell lines and primary bronchial epithelial cells had base-line constitutive expression of VCAM-1 surface protein and mRNA. These data are the first to demonstrate constitutive expression of VCAM-1 on respiratory epithelial cells and concord with the recent observations of Atsuta et al. (42), who found soluble VCAM-1 in supernatants from primary bronchial epithelial cells (51). These authors also observed inducible VCAM-1 surface expression in a transformed bronchial epithelial cell line (BEAS-2B) following stimulation by the combination of IL-1β and TNFα, although they were not able to demonstrate constitutive expression in this cell line. Maximal induction of surface expression and production of soluble VCAM-1 was observed with the combination of TNFα and IL-4 in BEAS-2B and primary cells, respectively (42). Our observations are different, in that we observed no significant effect on surface expression of VCAM-1 on A549 pulmonary or 16 HBE bronchial cell lines, of several proinflammatory cytokines or combinations of cytokines that are known to induce expression of several molecules including adhesion molecules in respiratory epithelial cells. Our studies included the combination of IFN-γ, IL-1β, and TNFα, at both 8 and 24 h, although we did not study the effect of IL-4 either alone or in combination. These data are of interest because they demonstrate that the regulation of VCAM-1 expression in respiratory epithelial cells is not readily inducible and suggest that the regulation of VCAM-1 expression may have unusual features that are not shared by other adhesion molecules that are easily up-regulated in respiratory epithelium.

It is therefore of great interest that we observed that rhinovirus infection induced increased VCAM-1 cell surface protein expression in each of A549 pulmonary and 16 HBE bronchial cell lines and in primary bronchial epithelial cells. The time course of induced expression was studied in A549 and 16 HBE cells and peaked in the former at 24 h and in the latter at 8 h after virus inoculation but in both cell lines remained elevated above noninfected cells for up to 72 h after inoculation.

We then investigated the group and serotype specificity of the induction of VCAM-1 and demonstrated that induction of VCAM-1 by rhinoviruses is clearly not receptor or serotype restricted. These observations are in keeping with previous observations relating to rhinovirus induction of IL-8 (26, 28, 29), IL-6 (27, 29), and ICAM-1 (44) and are important in that they suggest that the mechanisms involved in induction of VCAM-1 are likely to have broad applicability across all rhinovirus serotypes.

| Sequence of double-stranded oligonucleotides | Position in VCAM-1 5' region | Type of binding site | Position of binding motif | Mutated binding site |
|----------------------------------------------|-----------------------------|---------------------|--------------------------|---------------------|
| 5'-CTTATACATTCCAGTAAAGATAACCT              | -258/-232                   | GATA                | -254/-251                | -239/-236          |
| 5'-CTTATGATTCCAGTAAATCAGAAGCT              | -258/-232                   | mGATA               | -254/-251                | GATA -254/-251     |
| 5'-CCTTGAAGGGATTCCCTCC                    | -64/-45                     | NF-κB               | -64/-45                  | GATA -239/-236     |

| Sequence of double-stranded oligonucleotides | Position in ICAM-1 5' region | Type of binding site | Position of binding motif | Mutated binding site |
|----------------------------------------------|-----------------------------|---------------------|--------------------------|---------------------|
| 5'-GGGGCTCATCGCCTGGCCACCCTGGCTCC             | -227/-200                   | SPI                 | -206/-201                | -206/-201          |

**TABLE I**

Double-stranded oligonucleotides used in EMSA
In the present studies, we observed that each of sICAM and UV inactivation and filtering virus particles from the inoculum completely abrogated the observed VCAM-1 induction. We also confirmed the receptor specificity of the sICAM inactivation by demonstrating that preocating a minor group virus (rhinovirus 2) with sICAM had no effect on VCAM-1 up-regulation. These data suggest that in contrast to rhinovirus induction of ICAM-1 or IL-8, where some induction of protein synthesis appears to occur consequent upon virus-receptor binding (28, 44), the signal to up-regulate VCAM-1 expression occurs through processes associated only with viral replication. These data along with the observed differences in cytokine regulation (we have found no effect of any of IFN-γ, IL-1β, and TNFα on VCAM-1 expression, whereas ICAM-1 expression is known to be strongly up-regulated by all three), suggest that the regulation of the two adhesion molecules in respiratory epithelium is different with respect to a number of stimuli including rhinoviruses.

The ability of rhinovirus infection to up-regulate respiratory epithelial cells surface expression of VCAM-1 may have particular importance in the mechanisms of virus-induced asthma exacerbations. We have previously demonstrated that rhinovirus colds induce bronchial mucosal intraepithelial CD4+ and CD8+ lymphocytes and eosinophil infiltration, with a more persistent eosinophilia in asthmatic subjects (19). Epithelial expression of VCAM-1 is likely to play an important function in retaining both types of inflammatory leukocyte in respiratory epithelium by binding to its ligand the leukocyte αβ integrin VLA-4, which is expressed on both lymphocytes and eosinophils. In addition, binding of VCAM-1 to its integrin ligand on leukocytes activates these cells and leads to secretion of pro-inflammatory cytokines and mediators (5–8). A further recent study suggested an important role for VCAM-1 in promoting inflammation in asthma by demonstrating that inhibition of binding of VCAM-1 to its ligand VLA-4 markedly inhibited lymphocyte and eosinophil infiltration in an animal model of allergen-induced inflammation (30). These data make epithelial VCAM-1 a prime target for therapeutic intervention strategies for virus-induced asthma exacerbations.

Having demonstrated that rhinovirus infection of respiratory epithelial cells increased VCAM-1 surface protein expression, we investigated the effects of rhinovirus infection on respiratory epithelial cell VCAM-1 mRNA expression. We observed rhinovirus induction of VCAM-1 mRNA in pulmonary epithelial cells occurring within 1 h of virus inoculation, peaking at 8 h and lasting up to 24 h post virus inoculation; no studies were carried out beyond this time point. These data confirmed that as with VCAM-1 surface protein expression, rhinovirus induction of VCAM-1 mRNA was also consequent upon viral replication but not virus-receptor binding.

Having observed rhinovirus induction of both VCAM-1 protein and mRNA expression, we wished to determine whether rhinovirus infection of respiratory epithelial cells increased VCAM-1 expression by up-regulating VCAM-1 gene transcription. To investigate this possibility, we analyzed in vitro transcription of VCAM-1 mRNA in rhinovirus-infected and noninfected A549 cells. We observed clear induction of VCAM-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 induction of respiratory epithelial cells rapidly increased de novo transcription of VCAM-1 mRNA. Next we wished to determine the molecular mechanisms involved in rhinovirus induction of VCAM-1 mRNA transcription, because these mechanisms might identify
Rhinovirus infection of A549 respiratory epithelial cells does not induce binding of nuclear transcription factors to the SP1 site in the ICAM-1 promoter. The effect of rhinovirus infection on constitutive SP1 transcription factor binding to the −227/−200 portion of the ICAM-1 promoter (Table I) was assessed by EMSA. Nuclear extracts were prepared from uninfected (time 0) and rhinovirus 16-infected A549 cells at various time points (30, 60, and 90 min) after infection and incubated with radiolabeled −227/−200 ICAM-1 probe. Resolution of binding complexes was accomplished on 5% polyacrylamide gels. Representative radiograph of one of at least three separate experiments. Two retarded complexes binding to the −227/−200 portion of the ICAM-1 promoter were present at baseline conditions, and binding levels were not modified by rhinovirus infection. Confirmation that these complexes were composed of proteins binding to the SP1 site within the probe was carried out with appropriate competition experiments (data not shown).

Fig. 9. Rhinovirus infection of A549 respiratory epithelial cells does not induce binding of nuclear transcription factors to the SP1 site in the ICAM-1 promoter.

The VCAM-1 promoter contains several potential transcription factor-binding sites, of which NF-κB, GATA, IRF-1, AP-1, and SP1 have been implicated in induction of VCAM-1 gene transcription in response to various pro-inflammatory stimuli, including TNFα, lipopolysaccharide and cytokines (47, 55–58).

In the present study, we used reporter gene assays to investigate the effect of rhinovirus infection on VCAM-1 promoter activity and observed that rhinovirus infection of A549 epithelial cells strongly induced VCAM-1 promoter activity. We therefore used sequentially deleted VCAM-1 promoter constructs to determine which sites in the VCAM-1 promoter were functional in rhinovirus induction of VCAM-1 promoter activity. We observed that sequential deletion of the VCAM-1 promoter up to −258 base pairs from the transcription initiation site had no effect on the ability of rhinovirus infection to induce VCAM-1 promoter activity. Deletion of the promoter to −98 base pairs reduced the rhinovirus induction by approximately 50% and further deletion to −44 base pairs completely abrogated the rhinovirus induction. These data suggest that elements contained within the −258 to −44 region of the VCAM-1 promoter were necessary for rhinovirus-induced up-regulation of VCAM-1 promoter activity to occur.

In EMSA assays we observed clear induction by rhinovirus infection of proteins binding to labeled probes containing both the −254/−251 and the −239/−236 GATA-binding sites and to probes containing the consensus NF-κB-binding site present at both the −72/−63 and the −57/−48 sites within the VCAM-1 promoter. These experiments confirmed the induction by rhinovirus infection of proteins binding to either or both of the −254/−251 and −239/−236 GATA-binding sites and to either or both of the −72/−63 and −57/−48 NF-κB-binding sites within the VCAM-1 promoter.

Mutational analysis was therefore carried out with reporter gene assays to investigate the functionality of these transcription factor-binding sites within the VCAM-1 promoter in rhinovirus infected and uninfected A549 respiratory epithelial cells. We observed that mutation of the −254/−251 and −239/−236 GATA sites reduced the rhinovirus responsiveness of the VCAM-1 promoter by approximately 60%, confirming the necessity of these sites for full rhinovirus induction of VCAM-1 promoter activity in A549 cells to occur. Similar studies were also carried out with a VCAM-1 promoter/reporter construct mutated at the −72/−63 or −57/−48 NF-κB sites. Rhinovirus responsiveness was completely abolished by mutation of either the NF-κB sites, confirming that each of these sites is also necessary for rhinovirus induction of VCAM-1 promoter activity to occur.
Rhinovirus Infection Induces VCAM-1 via NF-κB and GATA

Previous studies have demonstrated that the NF-κB family of transcription factors are important in induction of pro-inflammatory cytokines by both rhinovirus and RS virus (32, 43, 45) and of ICAM-1 by rhinovirus (44). These data suggest that NF-κB may play a very important role in the induction of pro-inflammatory molecules by virus infections in general, and the data reported here extend these observations to include VCAM-1 induction by rhinoviruses.

Our demonstration that rhinovirus induction of VCAM-1 expression in respiratory epithelial cells is regulated by members of the GATA transcription family is entirely novel. The GATA family is known to be important in regulating hematopoiesis; GATA-1 is important in differentiation of erythroid cells, and GATA-3 is important in lymphocyte differentiation in particular.

GATA-3 is thought to have an important role in lymphocyte Th2 differentiation (59). Neish et al. (47) have reported involvement of members of the GATA family in regulation of VCAM-1 expression by TNFα in endothelial cells. Our data are the first to report regulation of any molecule by GATA in epithelial cells and also the first to report regulation of any molecule by virus infection in any cell type via the GATA family of transcription factors. These data suggest new functions for the GATA family of transcription factors that have not previously been recognized and that now merit further investigation.

In conclusion, we have demonstrated that rhinovirus infection of respiratory epithelial cells increases VCAM-1 surface expression via NF-κB- and GATA-mediated transcriptional up-regulation. We believe that VCAM-1 along with the NF-κB and GATA families of transcription factors represent new targets for potential therapeutic intervention in virus-induced asthma exacerbations.

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