Human Rhinovirus Selectively Modulates Membranous and Soluble Forms of Its Intercellular Adhesion Molecule-1 (ICAM-1) Receptor to Promote Epithelial Cell Infectivity*

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Human rhinoviruses are responsible for many upper respiratory tract infections. 90% of rhinoviruses utilize intercellular adhesion molecule-1 (ICAM-1) as their cellular receptor, which also plays a critical role in recruitment of immune effector cells. Two forms of this receptor exist; membrane-bound (mICAM-1) and soluble ICAM-1 (sICAM-1). The soluble receptor may be produced independently from the membrane-bound form or it may be the product of proteolytic cleavage of mICAM-1. The ratio of airway epithelial cell expression of mICAM-1 to the sICAM-1 form may influence cell infectivity and outcome of rhinovirus infection. We therefore investigated the effect of rhinovirus on expression of both ICAM-1 receptors in normal human bronchial epithelial cells. We observed separate distinct messenger RNA transcripts coding for mICAM-1 and sICAM-1 in these cells, which were modulated by virus. Rhinovirus induced mICAM-1 expression on epithelial cells while simultaneously down-regulating sICAM-1 release, with consequent increase in target cell infectivity. The role of protein tyrosine kinases was investigated as a potential mechanistic pathway. Rhinovirus infection induced rapid phosphorylation of intracellular tyrosine kinase, which may be critical in up-regulation of mICAM-1. Elucidation of the underlying molecular mechanisms involved in differential modulation of both ICAM-1 receptors may lead to novel therapeutic strategies.

Human rhinoviruses (HRV)† are the most frequent cause of upper respiratory tract infections known as the “common cold.” Although these infections are generally mild and self-limiting, they inflict a heavy economical burden due to high loss of productivity and medical costs (1). Currently, there is no effective treatment for HRV infections; over the counter cold remedies only alleviate the symptoms but do not eradicate the virus.

Primarily, HRV target epithelial cells for attachment and entry. These cells express intercellular adhesion molecule 1 (ICAM-1), the receptor for 90% of HRV serotypes (2). Both this major group of HRV and the 10% of HRV that use alternative receptors for cell attachment enhance cell surface ICAM-1 expression (3). This glycoprotein, belonging to the immunoglobulin supergene family, consists of five Ig-like domains (4); domains 1 and 2 have been shown to fit snugly in a key-lock relationship into reciprocal canyons on the HRV shell (5). In addition, to this critical role as a docking molecule during HRV infection, ICAM-1 through separate domains with its cognate ligand LFA-1 (CD18/CD11a) drives the migration of immune-effector cells to sites of inflammation (6). While most studies refer to the membranous form of ICAM-1, a soluble form (sICAM-1) has also been described (7). The molecular mass of sICAM-1 is similar to the molecular mass of the extracellular domain of ICAM-1 (80–114 kDa) depending on the level of glycosylation, suggesting that this soluble circulating form of ICAM-1 consists of most of the extracellular domain of membranous ICAM-1 (7). Several circulating isoforms of sICAM-1 have also been detected of 240, 430, and >500 kDa in size, indicating that sICAM-1 may circulate in a complexed form either with itself or with other proteins (7–8). While the exact origin of sICAM-1 is unclear, sICAM-1 may be produced directly from the membrane-bound form by proteolytic cleavage (9–10) or produced independently by an alternative splicing mechanism (11). What role is played by soluble ICAM-1 in disease pathogenesis remains to be elucidated.

Previous studies have demonstrated that pro-inflammatory cytokines can alter the expression of mICAM-1 (3, 12–14) and sICAM-1 (15–16). In addition, HRV infection has been shown to significantly up-regulate the expression of its membrane-bound receptor ICAM-1 on the surface of epithelial cells (3, 12–14) leading to an increase in epithelial cell infectivity (12). However, there is evidence indicating that sICAM-1 may have the opposite effect because it possesses antiviral properties both in vitro (17) and in vivo (18–19). Therefore, the dynamic inter-relationship between mICAM-1 and sICAM-1 forms may have a critical bearing on the pathogenesis as well as course of HRV infection. Thus, there is a need for a better understanding of the interaction between mICAM-1 and sICAM-1, leading to potential targets for therapeutic modulation of the course of HRV infection.

To investigate this hypothesis, a series of studies were undertaken to establish the presence of distinct mRNA transcripts coding for mICAM-1 and sICAM-1 in an in vitro airway epithelial cell model and to determine whether HRV has the
ability to modulate the two forms of ICAM-1 and how this affects epithelial cell infectivity. Having found that epithelial cells express the two ICAM-1 forms and that HRV could selectively modulate membrane and soluble ICAM-1 expression in an inverse fashion to promote/propagate infection, we explored the potential intracellular mechanisms influencing this differential modulation to identify potential targets for antiviral therapy.

**ICAM-1** is a rapid response gene with a complex pattern of regulation (20). Numerous second messenger signaling pathways involved in the activation of the ICAM-1 gene have been identified (20–21). A recent study demonstrated that HRV up-regulates membrane-bound ICAM-1 expression in airway epithelial cells via an NFkB-dependent mechanism (3). We investigated this intracellular signaling pathway further and examined the effect of HRV on tyrosine kinase phosphorylation in airway epithelial cells. In addition, we utilized inhibitors of gene transcription and protein synthesis to investigate potential mechanisms responsible for the modulation of sICAM-1 production/release during HRV infection.

Our results demonstrate that HRV selectively induces mICAM-1 expression on epithelial cells, at least in part, through a tyrosine kinase-dependent pathway, while, HRV influence on sICAM-1 release may involve the down-regulation/inhibition of proteolytic enzymes associated with the cleavage of mICAM-1 from the epithelial cell surface. The interaction of HRV with these intracellular molecular pathways controlling mICAM-1/sICAM-1 ratios will need to be further dissected, specifically for the development of new anti-HRV strategies aimed at either halting progression or reversing host cell infectivity.

**MATERIALS AND METHODS**

**Epithelial Cell Culture**—Normal human bronchial epithelial cells (NHBE) were obtained from Clonetics Corp., Walkersville, MD. The donor was a middle-aged male Caucasian. NHBE cells were cultured in small airway basal medium (SABM) supplemented with epidermal growth factor (25 ng/ml), hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), transferrin (10 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (0.5 ng/ml), bovine pituitary extract (25 μg/ml), retinoic acid (0.1 ng/ml), gentamicin (50 μg/ml), amphotericin B (50 μg/ml) at 37 °C in humidified air containing 5% CO2. All reagents were obtained from Clonetics Corp. In subsequent experiments, NHBE cells were seeded in 6-well plates at a density of 100,000 cells/well and utilized when 70–80% confluent.

**Viral Stocks**—The main rhinovirus seed (HRV-14) was kindly donated by J. Kent (University of Leicester). A stock solution of HRV-14 was generated by infecting confluent monolayers of HeLa Ohio cell line as described previously (Sethi et al., Ref. 12). Briefly, confluent monolayers of Hela cells were inoculated with a known dilution (10⁻⁴, TCID50/ml) of HRV-14 and incubated for 90 min at 34 °C in humidified air containing 5% CO2, after which, cells were cultured until the cytopathic effect (CPE) was >80%. Medium containing virus was centrifuged at 600 × g for 10 min, after which, the viral suspension was stored at −80 °C until required.

**Viral Purification**—Prior to use viral stocks were purified using a sucrose gradient. 20 μg/ml RNAse A (Sigma) was added to the viral suspension and incubated at 35 °C for 20 min. 1% sodium sarcosyl (Sigma) and 2-mercaptoethanol (1 μg/ml) were added to the RNAse-treated viral suspension. This was then overlaid on 1 ml of purification solution (20 mM Tris acetate, 1 mM NaCl, 30% w/v sucrose) and centrifuged at 200,000 × g for 5 h at 16 °C. The supernatant was discarded, and the resulting virus pellet was resuspended in medium and stored at −80 °C until required.

**HRV-14 Infection of Epithelial Cells**—Once cell monolayers were 70–80% confluent the culture medium was removed, and the cells were inoculated with HRV-14 10⁻⁴, TCID50/ml for 90 min at 34 °C, 5% CO2/air. The cells were washed, and maintenance medium was added to sustain cell growth. The gene and protein expression of the two ICAM-1 forms were measured simultaneously at 0, 8, 24, and 96 h postinfection. 0 h represents the point immediately after the 90-min inoculation period; subsequent time points (8–96 h) are taken from this 0 h.

**Measurement of mICAM-1 Protein Expression**—Membrane-bound HRV-14 expression was evaluated at 0, 8, 24, and 96 h post-HRV-14 infection. At each time point, cells from 6-well plates were collected via trypsinization and centrifugation; 6 cytoplasts for each experimental condition at each time point were prepared for immunostaining. The remaining cells were used for RNA extraction, cDNA synthesis, and reverse transcription (RT)-PCR. Internal controls consisting of unstimulated and uninfected cells were set up at each time point to allow comparisons between controls and treated cells. A 1:20 dilution of human immunoglobulin IgG-specific monoclonal antibody (Sigma) was used to confirm the epithelial origin of NHBE cell lines. Surface ICAM-1 was semiquantified using a 3-step indirect immunoenzymatic labeling method (22) and modified as described previously (12–13). Briefly, exogenous peroxidase staining was blocked using 2% bovine serum albumin/phosphate-buffered saline solution. NHBE cells were incubated with ICAM-1 monoclonal antibody at a concentration of 5 μg/ml (R1/1.1, IgG, Boehringer Ingelheim) at room temperature for 30 min. Cells were then washed using a washing buffer (Tris stock solution, 0.05 mol/liter, pH 7.4, NaCl, 0.9% saline solution), and incubated with rabbit anti-mouse IgG conjugated to peroxidase at a concentration of 1 mg/ml (Dako), then washed again. A third antibody, swine anti-rabbit IgG also conjugated to peroxidase, (Dako), was then added at a concentration of 0.8 mg/ml to amplify the staining intensity. The cells were then incubated with the substrate 3,3-diaminobenzidine tetrahydrochloride (0.6 mg/ml, Sigma) and stained with Mayer’s Hemalum solution (BDH). The cells were incubated with an antibody IgG antibody (Clone 1B2) at a concentration of 10 μg/ml, which acted as a negative control.

To avoid observer bias, the cytoplasts were scored by two independent observers (A. Bianco and S. Whiteman); a mean of three readings of each slide was performed. Two cytoplasts for each experimental condition per time point were assessed at ×400 magnification with a light microscope (Olympus CH-2 microscope, Olympus Optical Co., Ltd., Tokyo). 300 cells per microscopic field were counted and surface ICAM-1 on epithelial cells was assessed using a 5 point scoring scale based on the intensity of staining and appearance of the nucleus: 0, gray/brown; 1, light brown; 2, medium brown; 3, medium/dark brown; 4, dark brown; in grades 0–2 the nucleus appears well defined, in 3–4 the nucleus is partially or fully obliterated. The number of cells in each grade was then multiplied with the respective grade index, and the resulting values summed. The final result was expressed as the POX score (12–13, 22), defined as the difference between the sum of the specific and background staining: [(a × 0) + (b × 1) + (c × 2) + (d × 3) + (e × 4)]−value for the control slide = POX score, where each letter represents the number of cells scored in the respective grade. The coefficient of variability of the differences between the counts obtained from all slides by both observers was between 4 and 12%; and that between the two observers for each time point was less than 5%.

**Measurement of sICAM-1 Protein in Cell Culture Supernatants**—Soluble ICAM-1 expression was also evaluated at 0, 8, 24, and 96 h after HRV-14 infection. Internal controls consisting of uninfected and unstimulated cells were set up at each time point to allow comparisons between controls and treated cells. Cell culture supernatants retrieved from each experimental condition at each time point were assayed for soluble ICAM-1 using a commercially available ELISA kit (BioSource International, CA). The minimum detectable level of human soluble ICAM-1 (hsICAM-1) was ~0.04 ng/ml. 100 μl of undiluted cell culture supernatant or standard were utilized in the assay, which was performed in accordance with the manufacturer’s guidelines.

**RNA Extraction and cDNA Synthesis**—Total RNA was extracted from NHBE cells at 0, 8, 24, and 96-h postinfection using Trizol (In-nitrogen) according to the manufacturer’s guidelines, and cDNA was synthesized from 2 μg RNA using 2 μl of 5× First Strand cDNA synthesis containing 5 pmol oligo(dT) primer, 5× buffer (50 mM, pH 8.3, 75 mM KCl, 3 mM MgCl2), 0.5 mM dNTP mixture, 0.5 units of RNase inhibitors, and 200 units of MMLV reverse transcriptase; the total reaction volume was 20 μl. All cDNA synthesis reagents were obtained from Clontech. This was then incubated at 42 °C for 1 h after which reverse transcriptase was inactivated at 94 °C for 5 min. The cDNA was then diluted to a final volume of 100 μl and stored at −80 °C for RT-PCR.

**Detection of Membrane and Soluble ICAM-1 Gene Expression using RT-PCR**—Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a control for cDNA synthesis and RT-PCR. Primers used to detect ICAM-1 by RT-PCR were 5'-TGT GAC TCG GAG TCA CCGA and CAT GTG GCC CAT GAC GTC CAC CAC (antisense). Primers for the detection of mICAM-1 and sICAM-1 were based on those described previously by Wakatsuki et al. (11). The sequence of the forward primer used to detect mICAM-1 was 5'-CAA GGG GAG TGC ACC CCA GAG.
HRV Modulation of ICAM-1 Forms

GTG-3' and 5'-CAA GGG AGG TCA CCC GCG AGC C-3'. Both primers were used in combination with a common reverse primer with the following sequence 5'-TGC AGT GCC CAT TAT GAC TG-3'. These primer pairs encompass the transmembrane domain of ICAM-1. The primers used to detect HRV were CGG ACA CCC AAA GTA G (sense) and GCA CTT CTG TTT CCC C (antisense). The RT-PCR consisted of 25 pmol of primers, 200 μM dNTPs, 1.5 mm MgCl2, 5 μl 10× PCR buffer, and 2.5 units of AmpliTaq Gold (PerkinElmer Life Sciences) in a 50-μl reaction mixture in a thermal cycler (PTC 200 Peltier Thermal cycler) under the following conditions: 95°C for 12 min, 94°C for 1 min and 15 s (denaturation step), 60°C (G3PDH) or 65°C (ICAM-1) for 1 min and 15 s (annealing step) and 72°C for 1 min (extension step) for a total of 30 cycles (G3PDH) or 35 cycles (ICAM-1), after which a final extension step was performed at 72°C for 10 min. RT-PCR products were resolved using 3% metaphor agarose (Flowgen) gel in TBE buffer (89 mM Tris, 89 mM sodium borate, 2 mM EDTA, Sigma). Gels were visualized using ethidium bromide and UV light and analyzed densitometrically (Model GS-670, BioRad) using Molecular Analyst (version 1.5). Restriction endonucleases were used to confirm the size of the RT-PCR products of 45 and 57 base pairs, and soluble ICAM-1 RT-PCR products were digested at the site of the deletion to give 63 and 20 bp products. In addition, to confirm the presence of the 19-bp deletion, RT-PCR amplicons were sequenced using an ABI PRISM automated sequencer model 310.

Viral Titer Assay—The TCID50 method was used to calculate the concentration of the virus in cell culture supernatants at 0, 8, 24, and 96 h postinfection. Serial dilutions of cell culture supernatants were incubated in cell monolayers in 96-well plates for 5 days at 34°C in humidified air containing 5%CO2. The presence of cytopathic effect (CPE) in the wells was used to calculate the TCID50, using the Karber formula (12–14). Furthermore, cell infectivity was confirmed by detecting the presence of HRV RNA within the cell using RT-PCR (23).

Prevention of Virus-Receptor Binding—To confirm the changes observed in ICAM-1 expression were true effects of HRV and not due to soluble factors within the viral inoculum, separate studies were designed using anti-ICAM-1 monoclonal antibodies to block HRV attachment and subsequent infection. NHBE cell monolayers were washed and incubated with anti-ICAM-1 monoclonal antibodies (mAb) (RI/1.1 Boehringer Ingelheim) at separate concentrations of 4, 8, and 16 μg/ml for 1 h at 37°C under 5% CO2-humidified air (24). After which the anti-ICAM-1 mAb solution was removed, and cell monolayers were washed and inoculated with HRV-14 at a concentration of 105 TCID50/ml for 90 min at 34°C under 5% CO2-humidified air. At 90 min the viral inoculum was removed, cell monolayer washed, and maintenance medium was replaced. Gene expression of both ICAM-1 forms was evaluated using RT-PCR at 24 h, because this time point was previously shown to reflect an optimum response in gene expression. Corresponding viral titers were also measured.

Inhibition of de Novo Protein Synthesis—To test the level at which HRV-14 regulates the expression of both ICAM-1 receptors, separate cell cultures were preincubated with cycloheximide, an inhibitor of de novo protein synthesis at a concentration of 10 μg/ml for 2 h at 37°C in humidified air containing 5% CO2. Cycloheximide is widely used as an inhibitor of protein synthesis and has an effect at 10 μg/ml (25). The cell monolayers were then washed and infected with HRV-14 as described above. Membrane and soluble ICAM-1 protein levels were assessed as described above.

Inhibition of Gene Transcription—Actinomycin D, an inhibitor of gene transcription was used to assess the effect of HRV-14 on ICAM-1 gene transcription. NHBE cells were incubated with actinomycin D at a concentration of 10 μg/ml for 2 h at 37°C in humidified air containing 5% CO2. 10 μg/ml was identified as the optimum dose in previous dose response experiments (data not shown). The cell monolayers were then washed and infected with HRV-14 as described above. Membrane-bound and soluble ICAM-1 were assessed using RT-PCR and semiquantified using densitometry (Model GS-670, BioRad) and Molecular Analyst Software (version 1.5).

Investigation of the Role of Tyrosine Kinases in HRV Induction of ICAM-1 Expression—The involvement of tyrosine kinase in the HRV-driven up-regulation of ICAM-1 gene expression was investigated using Western blot. NHBE cells were cultured in SABM (detailed under “Materials and Methods”) and infected with HRV-14 (TCID50, 105 for 2 h at 37°C) and then infected with HRV-14 (TCID50, 105 for the next 10–20 min, 0 min represents the viral inoculum placed on the cell monolayer and then immediately removed). Cell were also pretreated with genistein (50 and 100 μM), an inhibitor of tyrosine kinase for 1 h at 37°C in 5% CO2-humidified air. NHBE cells were lysed using a lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl, pH 7.5 and 150 μM of reduced protein samples were electrophoresed on 12.5% SDS-PAGE and transferred to nitrocellulose membranes (sandwiches, 0.45-μm pore size Novex, San Diego). Molecular weight markers and epidermal growth factor receptor (control) were run with the samples. Membranes were blocked with 10% (w/v) low fat milk for 1 h in TBS-T and probed for 2 h with mouse anti-human phosphotyrosine kinase (clone 4G10, Upstate Technology) diluted 1:3000 in TBS-T. Membranes were incubated with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako) diluted 1:40,000 in TBS-T and then 25 μg of reduced protein samples were electrophoresed on 12.5% SDS-PAGE and transferred to nitrocellulose membranes (sandwiches, 0.45-μm pore size Novex, San Diego). Molecular weight markers and epidermal growth factor receptor (control) were run with the samples. Membranes were blocked with 10% (w/v) low fat milk for 1 h in TBS-T and probed for 2 h with mouse anti-human phosphotyrosine kinase (clone 4G10, Upstate Technology) diluted 1:3000 in TBS-T. Membranes were incubated with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako) diluted 1:40,000 in TBS-T and then probed for 2 h with mouse anti-human phosphotyrosine kinase (clone 4G10, Upstate Technology) diluted 1:3000 in TBS-T. Membranes were incubated with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako) diluted 1:40,000 in TBS-T and then probed for 2 h with mouse anti-human phosphotyrosine kinase (clone 4G10, Upstate Technology) diluted 1:3000 in TBS-T.

Statistical Analysis—Each experiment was performed three times. Data were expressed as means ± S.E., and comparisons between experimental conditions and controls were performed by paired Student’s t test. Probability values < 0.05 were considered significant.

RESULTS

Epithelial Cell Expression of Two Distinct Forms of mRNA Coding for ICAM-1—Two distinct mRNA transcripts were observed in NHBE cells after RT-PCR (Fig. 1). These RT-PCR products corresponded with those observed by Wakatsuki et al. (11) with RT-PCR products of 102 and 83 bp for mICAM-1 and sICAM-1, respectively. Product sizes were confirmed by restriction enzyme digestion (data not shown).

Influence of HRV-14 on mICAM-1 Expression—mICAM-1 surface protein was constitutively expressed in NHBE cells (Fig. 2A). HRV-14 infection induced an up-regulation in both surface mICAM-1 protein and gene expression (Fig. 2A and B). Surface mICAM-1 protein expression on NHBE cells increased by 2.5-fold over baseline at 8 h post-HRV-14 infection (*, p < 0.001, Fig. 2A). The enhanced mICAM-1 protein levels were sustained throughout the 96-h period of infection and was accompanied by a 2-fold increase in mICAM-1 gene expression (normalized to the housekeeping gene G3PDH) only at 24 h through to 96 h (*, p = 0.02, Fig. 2B).

Influence of HRV-14 on sICAM-1 Expression—Basal sICAM-1 protein release in cell culture supernatants increased at 8 h...
and thereafter decreased in a time-dependent manner. However, no sICAM-1 protein was detected in cell culture supernatants retrieved from HRV-infected cell lines at any time point studied. Gene analysis showed that HRV-14 infection induced a persistent down-regulation in sICAM-1 gene expression in NHBE cells over the study period (0–96 h); with sICAM-1 mRNA levels decreasing to half-basal mRNA levels at 24 h (*, *p < 0.001, Table I). Fig. 1 illustrates the simultaneous changes in membrane and soluble ICAM-1 gene expression during HRV infection of NHBE cells.

**Prevention of Virus-Receptor Binding**—In order to confirm that the observed changes in epithelial cell membrane and soluble ICAM-1 expression, reflected the direct influence of HRV-14, separate blocking experiments were carried out using anti-ICAM-1 mAb. NHBE cultures were incubated with ICAM-1 mAb prior to HRV-14 infection. mICAM-1 and sICAM-1 gene expression were evaluated 24-h post-HRV-14 infection. Viral titers in retrieved cell culture supernatants were also measured. Incubation of control uninfected cultures with ICAM-1 mAb had no significant effect on the gene expression of mICAM-1 and sICAM-1 compared with equivalent untreated cultures (data not shown). Preincubation with ICAM-1 mAb at 4 μg/ml resulted in a decrease in HRV-14-induced gene expression of mICAM-1 (*, *p < 0.02 Fig. 4A) but had no significant effect on the gene expression of sICAM-1. Preincubation with 8 μg/ml and 16 μg/ml
ICAM-1 mAb also resulted in a decrease in mICAM-1 expression. In addition, these higher concentrations also produced small but significant increases in sICAM-1 expression (*, p < 0.05 compared with mICAM-1 expression of untreated infected cells; **, p < 0.02 compared with sICAM-1 expression of untreated infected cells). Viral titers in recovered cell culture supernatants were measured using the TCID_{50} and are expressed as a log TCID_{50}/ml. Data are mean ± S.E. of three separate experiments (*, p < 0.02 compared with viral titers from infected untreated cells).

**Inhibition of de Novo Protein Synthesis**—Treatment of NHBE cells with cycloheximide (10 µg/ml) resulted in an inhibition of HRV-induced mICAM-1 protein expression at 0 and 8 h, suggesting that HRV-14 induces de novo protein synthesis of mICAM-1. However cycloheximide had no significant effect on mICAM-1 protein expression at 24 and 96 h (data not shown). This may be caused by the depletion of cycloheximide in the culture medium as it has a short half-life. In contrast, sICAM-1 release from the same HRV-infected NHBE cells was not affected by cycloheximide, implying that the regulation of sICAM-1 release from infected cells is not dependent on de novo protein synthesis.

**Inhibition of Gene Transcription**—Treatment of NHBE cells with actinomycin D (10 µg/ml) resulted in a complete inhibition of the expected HRV-induced mICAM-1 protein expression at 8, 24, and 96 h (*, p < 0.001, Fig. 5A). In contrast, actinomycin D enhanced sICAM-1 protein release from HRV-infected cells at all experimental time points post-HRV inoculation. These observations indicate that HRV may down-regulate sICAM-1 release by inhibiting gene transcription of a suppressor/inhibitor of an enzyme responsible for the cleavage of mICAM-1 (*, p < 0.001, Fig. 5B).

**Investigation of the Role of Tyrosine Kinases in HRV Induction of ICAM-1 Expression**—Previous studies have demonstrated that the malarial parasite *Plasmodium falciparum* utilizes and up-regulates ICAM-1 expression via a tyrosine kinase-dependent mechanism (26). We therefore sought to determine whether HRV-14 also acts on ICAM-1 expression via this mechanism. Phosphorylation of tyrosine kinase was assessed using Western analysis. HRV-14 infection of NHBE cells induced the de novo phosphorylation of a number of cellular substrates (Fig. 6A). Densitometry of three separate blots was performed, and the data are expressed as a percentage increase in tyrosine kinase phosphorylation in HRV-infected cells compared with...
control uninfected cells at each time point. The predominant substrates included a band at 85 kDa (Fig. 6B) and at 200 kDa (Fig. 6C). HRV-14 induced a rapid increase in tyrosine kinase phosphorylation occurring within 5 min of infection, where tyrosine kinase phosphorylation of the 85- and 200-kDa substrates increased by 80 and 120%, respectively, compared with uninfected to control uninfected cells (*, \(p < 0.01\)). This activation of the 200-kDa substrate was reduced by 30-min post-viral inoculation, while the 85-kDa protein remained activated up to 30 min (*, \(p < 0.05\) at 5 min compared with 15 and 30 min). The observed HRV-induction of tyrosine kinase phosphorylation was completely inhibited by preconditioning cells with 50 and 100 \(\mu M\) genistein (data not shown). In addition, incubation of these NHBE cells with genistein down-regulated the HRV-induced increase in mICAM-1 protein on the cell surface (*, \(p < 0.001\), Fig. 7).

**DISCUSSION**

In this present study, we have investigated the mechanisms driving the regulation of the major rhinovirus group receptor, ICAM-1 at the epithelial cell level during infection. As ICAM-1 can exist in two distinct forms, which appear to have opposing influences on host cell infectivity, we postulate that the dynamic inverse relationship between the membranous and soluble ICAM-1 receptor types is a critical catalyst in the pathogenesis and outcome of HRV infection.

**FIG. 6.** Western blot of phosphorylated tyrosine kinase activity in control uninfected NHBE cells and HRV-infected cells over a time course. \(M\) represents the molecular weight marker; control cells at 0 min (lane 1), infected cells at 0 min (lane 2), infected cells at 5 min (lane 3), control cells at 5 min (lane 4), control cells at 15 min (lane 5), infected cells at 15 min (lane 6), infected cells at 30 min (lane 7), and control cells at 30 min (lane 8). Time 0 min represents the viral inoculum placed onto the cell monolayer and then immediately removed; subsequent time points are taken from this point. This blot is typical of three separate experiments (A). Phosphorylated tyrosine kinase activity was analyzed by Western blotting and semi-quantified using densitometry. Densitometry analysis revealed 2 prominent bands at 85 kDa (B) and 200 kDa (C). Data represent the mean density reading from three separate Western blots corrected for background ± S.E. and expressed as a percentage increase relative to control uninfected cells at each time point (*, \(p < 0.01\) 5 min compared with 0 min; **, \(p < 0.05\) at 5 min compared with 15 and 30 min).

**FIG. 7.** Effect of genistein (100 \(\mu M\)) on the HRV-14 induction of mICAM-1 protein expression on bronchial epithelial cells at 0–96 h. mICAM-1 protein expression was measured using immunocytochemistry and expressed as a Pox score. Data are mean ± S.E. of three separate experiments (*, \(p < 0.001\) compared with untreated HRV-14-infected cells). HRV-infected cells are represented by black bars and HRV-infected cells preincubated with genistein are represented by gray bars.

We have demonstrated the presence of distinct mRNA transcripts coding for membrane-bound and soluble ICAM-1 in airway epithelial cells. We have also shown that these two
receptors may be regulated independently. Consistent with previous studies (3, 12–14), our experiments demonstrated that rhinovirus infection of NHBE cells, increased the expression of mICAM-1 at both the protein and gene level peaking in this study at 8 and 24 h after virus inoculation, respectively, and thereafter, remained elevated above comparative uninfected control cells for up to 96 h after inoculation.

In addition, we explored effects of HRV on sICAM-1 gene and protein expression on the above cells. Rhinovirus appeared to down-regulate the gene expression of sICAM-1 throughout the study period reaching half-basal levels of expression by 24 h. This observed down-regulation of sICAM-1 mRNA is supported by the absence of detectable sICAM-1 in cell culture supernatants retrieved from HRV-infected cells. As virus and the antibody applied in the sICAM-1 ELISA utilize the same binding site on sICAM-1 molecules, the possibility of interference from HRV with the ELISA was eliminated by assaying controls “spiked” with recombinant sICAM-1 (data not shown).

To our knowledge, we are the first to report the simultaneous differential effect of HRV on both membrane and soluble forms of ICAM-1 receptor in an epithelial cell model, such that the virus appears to induce the membrane-bound form, while dramatically decreasing the soluble component; thereby facilitating and promoting cell infectivity. Of particular interest, we have conducted other studies, which show this same pattern of inverse relationship between mICAM-1 and sICAM-1 pattern of regulation following HRV inoculation of other epithelial cell lines, BEAS-2B and H292 (data not shown) (27). However, these studies also suggest that differences can occur in the time kinetics and magnitude of responses dependent on cell type used. Indeed, this is supported by the observations of Papi and Johnston (3), who observed significant magnitude differences in viral-induced ICAM-1 cell surface expression between primary bronchial epithelial cells and A549 (bronchial carcinoma-derived) epithelial cells. Thus direct comparison of data between studies needs to take into account differences in cell origin, culture techniques as well as assay conditions used, as all these factors may account for observed differences in responsiveness to HRV infection.

Taken together, these results suggest that sICAM-1 may be detrimental to the virus as its competitive binding to available virus particles would facilitate a defensive role in limiting viral infection of target cells. Indeed, a recombinant form of sICAM-1 has been shown to have inhibitory effects on HRV infection in vitro (17). Studies in chimpanzees (18) and humans (19) have shown recombinant sICAM-1 to have some prophylactic effects with reduced severity of experimental rhinoviral colds. A recent in vivo study investigated mICAM-1 expression on nasal scrape biopsies and sICAM-1 levels in nasal lavage fluid from separate volunteer groups inoculated with experimental rhinovirus (28). mICAM-1 expression was increased in 87% of the volunteers following infection with rhinovirus; however, sICAM-1 levels in the nasal lavage fluid were only increased in 47% of volunteers. This differs from our current study; we found no detectable sICAM-1 in cell culture supernatants from HRV-infected cells. This apparent discrepancy in results may be caused by fundamental differences in the study design as our study is an in vitro study using cell lines. In addition, as less than half the volunteers exhibited an increase in sICAM-1 in the nasal lavage fluid, it is equally possible that the sICAM-1 measured may be due to mLICAM-1 diglosed from the cell surface as a result of the sampling process and not through direct release. Time kinetics of ICAM-1 induction was consistent with our study in that up-regulation of ICAM-1 occurred within 24 h of infection and declined by day 5 (28).

Levels of infectious virus in cell culture supernatants from infected cells increased 8-h postinoculation and remained elevated for up to 96 h, suggesting an increase in viral replication over time. These data were confirmed by RT-PCR, in which a time-dependent increase in HRV RNA within NHBE cells was observed.

Blocking viral binding and subsequent viral internalization using monoclonal antibodies against ICAM-1 resulted in the inhibition of both the HRV-induced increase in mICAM-1 and down-regulation of sICAM-1 expression. In addition, viral titers in retrieved cell culture supernatants were significantly lower in cells pretreated with ICAM-1 monoclonal antibodies, suggesting a reduction of initial viral binding, entry, and subsequent infection. These data suggest that the observed HRV effects on ICAM-1 expression are due to virus specific-epithelial cell receptor interactions. This information may facilitate the design of potential small molecule therapeutic inhibitors targeting HRV-cell receptor interactions or subsequent intra-cellular events following viral binding and release of genetic material into the cell.

The HRV-induced increase in mICAM-1 expression was inhibited by cycloheximide indicating that HRV initiates de novo protein synthesis of ICAM-1. However, cycloheximide had no effect on sICAM-1 release from HRV-inoculated cells. Treatment of NHBE cells with actinomycin D also inhibited the HRV-induced increase in mICAM-1 suggesting HRV initiates transcription of the ICAM-1 gene. In contrast, actinomycin D increased the release of sICAM-1 from HRV-inoculated NHBE cells, suggesting HRV may increase transcription of a suppressor/ accessory protein preventing the activity of a proteolytic enzyme involved in the cleavage of mICAM-1 from the cell surface. Possible candidate enzymes involved in the cleavage of mICAM-1 include the metalloproteinases (MMPs), which are tightly regulated by tissue inhibitors of metalloproteinases (TIMPs). In human gastric adenocarcinoma cells, Helicobacter pylori has been demonstrated to modulate MMP and TIMP secretion and that host MMP-3 and a TIMP-3 homolog expressed by H. pylori mediate at least in part of the host cell response to infection (29). A similar mechanism may operate in HRV-infected bronchial epithelial cells. In addition, a study utilizing astrocytes demonstrated that the mechanism involved in sICAM-1 release was sensitive to metalloprotease inhibitors (30). Proteolytic cleavage of mICAM-1 has also been observed in keratinocytes, where the addition of protease inhibitors resulted in a dose-dependent inhibition of sICAM-1 production (31). It is therefore plausible that HRV may modulate the release of sICAM-1 by both down-regulating the gene expression of sICAM-1 and manipulating the potential enzymatic reactions involved in the cleavage of mICAM-1 from bronchial epithelial cells. Further studies need to identify enzymatic pathways responsible for the cleavage of mICAM-1 in bronchial epithelial cells.

HRV has been shown to inhibit nuclear import, thus preventing signal transduction into the nucleus (32). This could serve as one pathway utilized by HRV during the down-regulation of sICAM-1 expression. Alternatively, expression of sICAM-1 protein may be blocked at the level of translation. Previous studies have demonstrated that certain viruses may block translation of mRNA at the initiation step (33). Indeed, a study conducted by Svitkin et al. (34) demonstrated that HRV inhibits host cell protein synthesis by cleaving the eukaryotic initiation factors eIF4G1 and eIF4G1 resulting in a 60% decline in host protein synthesis by 6 h. This mechanism may contribute to the HRV-induced inhibition of sICAM-1 release as no sICAM-1 protein was detected at 0 h (8 h after inoculation with HRV-14). Translation termination factors may serve as a target for the virus. It has been demonstrated that certain isolated RNAs have an
affinity for eukaryotic translation termination factors, eRF1, and eRF1-eRF3 complexes; to which they not only bind but also inhibit eRF1-mediated release of protein precursor chains from ribosomes (35).

Furthermore, HRV could modulate sICAM-1 secretion. Studies have demonstrated that poliovirus, also a member of the picornaviridae family, inhibits the transport of both plasma and secretory proteins from the endoplasmic reticulum to the Golgi apparatus early in the infection cycle (36–37). It is therefore plausible that the above pathways, either solely or partly in combination could drive the observed HRV-induced down-regulation in sICAM-1 release.

Since our studies have shown an HRV-induced increase in mICAM-1 expression at the transcriptional level, we proceeded to investigate the potential molecular mechanisms involved. Previous studies have shown that HRV induced up-regulation of mICAM-1 gene promoter activity involves initiation of NFκB proteins binding to the NFκB binding site on the ICAM-1 gene promoter region (3). Other studies have also indicated that tyrosine kinases may play a role in the regulation of the ICAM-1 gene (26, 38–39). We have examined this intracellular pathway further and demonstrated that HRV initiates rapid onset of tyrosine phosphorylation of multiple substrates. There was strong phosphorylation of two substrates, 85 and 200 kDa, 5 and 15 min post-HRV inoculation. This response was totally inhibited by genistein at concentrations of 50 and 100 µM. Kelley and Drumm (40) also demonstrated that ICAM-1 expression was mediated through tyrosine kinases of 85 and 154 kDa proteins binding to the NFκB binding site on the ICAM-1 gene promoter (41). Huber et al. (41) concluded that this protein was of cellular origin and may play a critical role in effective viral replication. In addition, tumor necrosis factor (TNF)-induced ICAM-1 expression has been demonstrated to involve the tyrosine phosphorylation of an 85-kDa protein, which was thought to be a cytoskeletal protein (41). Further studies utilizing more specific inhibitors for example herbimycin A, a selective inhibitor of Src-like kinases or tyrphostin, an inhibitor of Janus kinase (JAK) are required to identify these proteins. In addition, specific monoclonal antibodies to antibodies to proteins of a similar molecular weight may be utilized to identify these substrates. In this study, genistein (100 µM) significantly inhibited the HRV-induced up-regulation in mICAM-1 expression at 8 h with levels decreasing to basal levels of expression by 96 h. These data support the hypothesis that HRV may modulate mICAM-1 expression through a tyrosine kinase-dependent signaling pathway.

In conclusion, we have demonstrated that HRV manipulates the expression of both ICAM-1 receptors in airway epithelial cells to promote and sustain infection. We have attempted to elucidate the complex molecular mechanisms of ICAM-1 regulation and identified protein tyrosine kinases as critical components. It is plausible that detailed dissection of the molecular driving forces involved in coordinating the inverse relationship between membranous and soluble ICAM-1 receptors in the context of HRV-epithelial cell membrane interaction may lead to the development of novel anti-HRV therapeutic strategies.