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Blocking Intercellular Adhesion Molecule-1 on Human Epithelial Cells Decreases Respiratory Syncytial Virus Infection

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The respiratory syncytial virus (RSV) causes potentially fatal lower respiratory tract infection in infants. The molecular mechanism of RSV infection is unknown. Our data show that RSV colocalizes with intercellular adhesion molecule-1 (ICAM-1) on the HEp-2 epithelial cell surface. Furthermore, a neutralizing anti-ICAM-1 mAb significantly inhibits RSV infection and infection-induced secretion of proinflammatory chemokine RANTES and mediator ET-1 in HEp-2 cells. Similar decrease in RSV infection is also observed in A549, a type-2 alveolar epithelial cell line, and NHBE, the normal human bronchial epithelial cell line when pretreated with anti-ICAM-1 mAb prior to RSV infection. Incubation of virus with soluble ICAM-1 also significantly decreases RSV infection of epithelial cells. Binding studies using ELISA indicate that RSV binds to ICAM-1, which can be inhibited by an antibody to the fusion F protein and also the recombinant F protein can bind to soluble ICAM-1, suggesting that RSV interaction with ICAM-1 involves the F protein. It is thus concluded that ICAM-1 facilitates RSV entry and infection of human epithelial cells by binding to its F protein, which is important to viral replication and infection and may lend itself as a therapeutic target.

Key Words: **normal human bronchial epithelial cells (NHBE); human type 2 alveolar epithelial cells (A549); HEp-2 cells; respiratory virus; F protein; adhesion molecule.**

The respiratory syncytial virus (RSV) is an important respiratory pathogen that produces an annual worldwide epidemic of respiratory illness primarily in children, but also in the elderly (1, 2). In the United States alone, RSV infection of children causes about 100,000 hospitalizations and 4500 deaths annually. RSV commonly precipitates bronchiolitis and exacerbates asthma but is also associated with severe life-threatening respiratory infections in individuals with coronary artery disease or who are immunocompromised (3–6).

RSV is a nonsegmented *Pneumovirus* with a negative-strand RNA genome. *Pneumoviruses* attach to the cell surface receptor through their attachment protein, G; however, a SH/G deletion mutant of RSV is able to infect cells (7–10). Antibodies to either the G or F protein neutralize virus infectivity, *albeit* anti-G antibodies have poor neutralizing ability compared to the anti-F antibodies, both in humans and mice (11, 12). Airway epithelial cells and macrophages are the primary targets for RSV infection; however, the mechanism of infection process, particularly the role of host cell in this process has been poorly studied. Cellular glycosaminoglycans, specifically iduronic acid containing heparin and heparan sulfate, play a role in mediating attachment of RSV to the cells by interaction with G and/or F proteins (13–15). It has also been reported that the RSV-F protein also binds to RhoA, which is necessary for syncytia formation (16). Furthermore, surfactant protein A was shown to bind to RSV attachment protein G and was implicated in facilitating RSV internalization into macrophage cells (17).

RSV infection increases expression Intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin gene superfamily, on epithelial cells (18, 19). Increased expression of cytokines and ICAM-1 induced by RSV infection on epithelial cells is similar to that induced by rhinovirus, which utilizes ICAM-1 is the major cell surface receptor (20–22). Moreover, the ICAM-1 cross-linking of human epithelial cells induced IL-1β mRNA, which is also rapidly induced by RSV infection of Hep-2 cells, suggesting involvement of ICAM-1 in the process (23). We therefore hypothesized that ICAM-1 may play a role in RSV infection and

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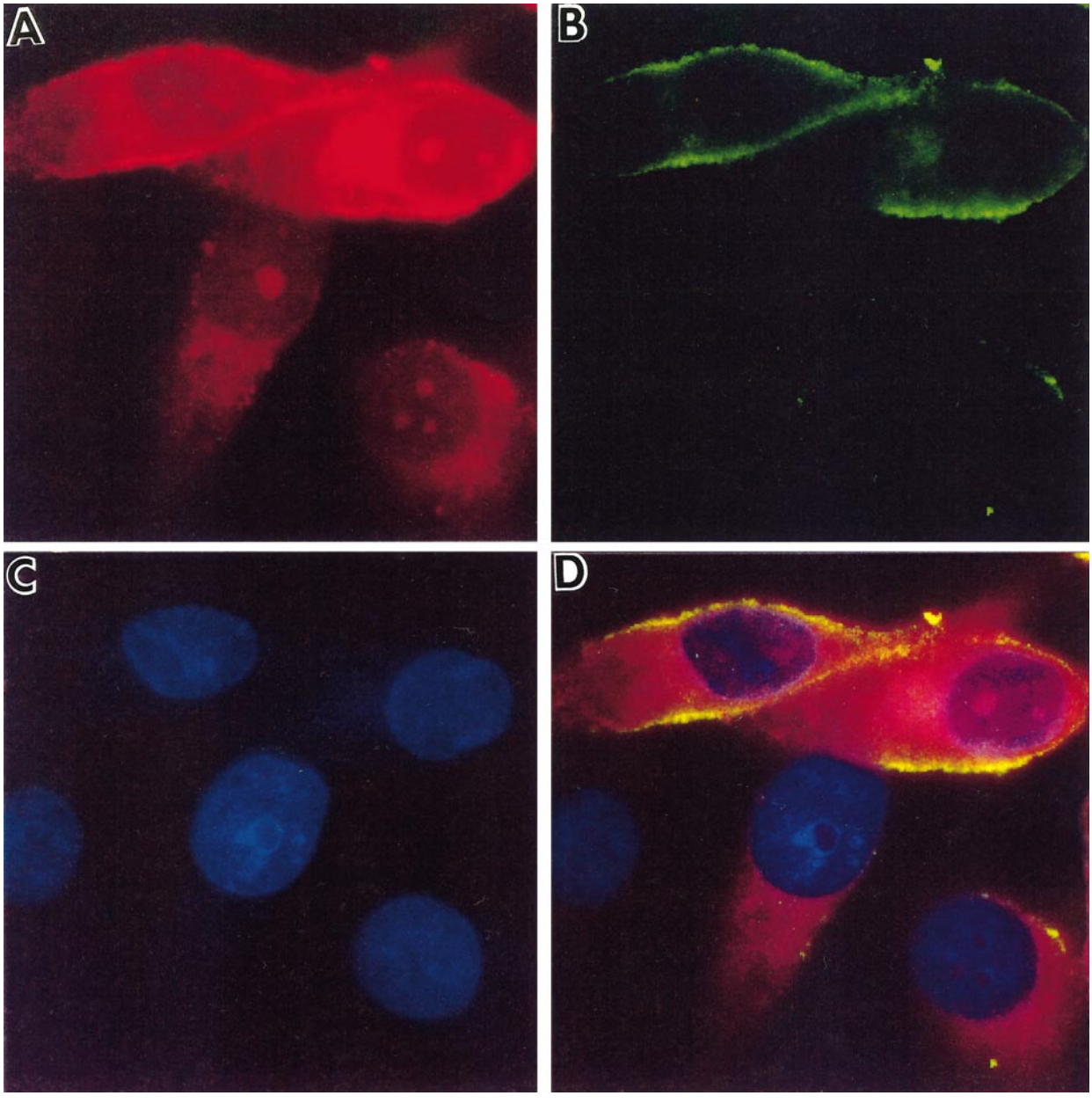

FIG. 1. Topography of ICAM-1 expression in RSV-infected HEp-2 cells by confocal microscopy as described. (A) RSV was distributed on the cell surface and in the cell cytoplasm (red) (B) ICAM-1 was localized mostly on the cell surface by FITC staining (green), (C) nuclei were visualized by staining with DAPI (blue). (D) Superimposition of all the three images indicated the colocalization of RSV and ICAM-1 on the plasma membrane of RSV-infected cells.

examined the effect of ICAM-1 blocking on RSV infection in this study. Our results demonstrate that ICAM-1 and RSV are colocalized on the plasma membrane of HEp-2 cells, and that ICAM-1 specifically binds to the F protein. Blocking of ICAM-1 on epithelial cells by anti-ICAM-1 mAbs reduces RSV infection.

MATERIALS AND METHODS

Cell culture, virus and infection. HEp-2 (ATCC CCL-23) cells which represents an epithelial-like cervical carcinoma cell line and RSV A2 Long strain (VR-1302) were obtained from the ATCC (Rockville, MD). HEp-2 cells were grown at 37°C with 5% CO_2 in a minimum essential medium with Hank's salt, supplemented with 10% fetal bovine serum (FBS). Viral stocks were prepared from infected HEp-2 cells after 5–6 days post infection (p.i.), stored at −70°C in aliquots and used as the viral inoculum. HEp-2 cells were infected with RSV at a moi of 1 and left for adsorption for 2 h at 37°C with 5% CO_2, after which the virus inoculum was replaced by complete medium. Cells were harvested at various hours post infection as specified for individual experiment.

Confocal microscopy. For confocal microscopic analysis, RSV infected HEp-2 cells grown on coverslips for 72 h were fixed in ethanol,

blocked for 1 h with 1% BSA in PBS, pH 7.4 and double stained with goat anti-RSV polyclonal Abs and mouse anti-human ICAM-1 mAb (BBA-4) each at 4 μg/ml (R&D Systems, Minneapolis, MN) for 1 h at 37°C. The cells were subsequently incubated for 1 h with secondary antibodies: rabbit anti-goat IgG-PE conjugate and sheep anti-mouse IgG-FITC conjugate, and were finally mounted in DAPI antifade (Oncor, Gaithersburg, MD). The cells were scanned using an Oncor digital confocal microscope at 525nm for FITC, at 580 nm for PE, at 350 nm for DAPI for counter staining of DNA; all three images were merged to confirm the colocalization of ICAM-1 and RSV in the same cell.

Prevention of RSV binding to ICAM-1 on cell surface. Viruses were precoated with excess soluble ICAM-1 (sICAM-1) to saturate the ICAM-1 binding sites on the virus capsid. Virus stock solutions were preincubated with sICAM-1 (R&D Systems, Minneapolis, MN) at various concentrations (0.1 to 100 μg/ml) for 30 min at room temperature and then used for infection. BSA was used as a nonspecific protein control and similarly preincubated with RSV.

Blockade of ICAM-1 in vitro. HEp-2 cells (10^5 cells/ml) were treated with different concentrations (100 to 400 μg/ml) of anti-ICAM-1 mAb (BBA 4) (R&D Systems, Minneapolis, MN) or purified mouse IgG_1 antibodies (isotype control) (Sigma, St. Louis, MO) for 3 h at 37°C and were subsequently infected with RSV at a moi of 1. Cells were harvested 24 h post-infection. The inhibition of RSV infection was determined by immunofluorescence, plaque assay and RT-PCR.

Immunofluorescence. RSV infected or uninfected cells were fixed in chilled acetone for 10 min and air-dried. Cells were stained for 30 min at 37°C with FITC-labeled anti-RSV mAbs (Chemicon, Temecula, CA) in a humid chamber. The unbound antibodies were removed by washing three times in PBS-Tween 20 (0.2%) buffer, pH 7.4. The slides were air-dried again and mounted on Fluromount G (Fisher, Pittsburgh, PA) and observed under fluorescent microscope. RSV positive cells (green fluorescence) were counted randomly from 15 different spots and from 2 different slides for the same treatment group and the percentage of infected cells was plotted against the concentration of ICAM-1 mAb or sICAM-1 used.

Semiquantitative reverse transcription PCR analysis. The total RNA was isolated from the harvested cells and tissue samples using Trizol (Life Tech., Gaithersburg, MD). Random or gene specific oligonucleotide primed cDNA was prepared using Superscript II RNase H^- reverse transcriptase (Life Tech., Gaithersburg, MD. The first strand cDNA product (1 μl) was amplified using *Taq* polymerase (Life Tech., Gaithersburg, MD). Forward and reverse primers used are as follows: RSV-N forward: 5′-GCG ATG TCT AGG TTA GGA AGA A-3′; reverse: 5′-GCT ATG TCC TTG GGT AGT AAG CCT-3′; ICAM-1 forward: 5′-ATG GCT CCC AGC AGC CCC-3′; reverse: 5′-CAC CTG GCA GCG TAG GGT-3′and β-actin forward: 5′-CGC GAG AAG ATG ACC CAG-3′; reverse: 5′-ATC ACG ATG CCA GTC GTA C-3′. All PCRs were denatured at 95°C for 1 min, annealed at 56°C for 1 min and extended at 72°C for 1 min for 22–35 cycles (depending upon the linear range). All amplifications were done in triplicate and repeated three times and a representative sample is shown. All amplifications were RNA specific, as no bands were seen in the control (no template) PCR samples. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and quantified using the advanced quantifier software (BioImage, Ann Arbor, MI).

RSV plaque assay. HEp-2 cells were seeded on 24 well plates. Cells were infected with 0.5 ml of RSV at various dilutions for 2 h at 37°C. Then the RSV was removed and the wells were overlaid with 1 ml of the growth medium containing 0.8% methylcellulose. The cells were incubated at 37°C for 72 h, after which the overlay was removed. Cells were then fixed in 80% cold methanol for 3 h. The methanol was removed and the plate was blocked with PBS and horse serum at 37°C for 30 min. Then the blocking solution was removed and mouse monoclonal anti-RSV antibody (NCL-RSV 3, Vector Laboratories, Burlingame, CA) diluted at 1:400, was added and incubated at 37°C for 1 h. Secondary antibody staining and substrate reactions were performed using the Vectastain ABC Kit (Vector Laboratories). Diaminobenzidine in H_2O_2 (Pierce, Rockford, IL) was used as a chromogen. The plaques were enumerated by microscopy.

ELISA for RSV-ICAM-1 Interaction. Soluble ICAM-1 protein (R&D Systems, Minneapolis, MN) or Bovine serum albumin, each at 100 ng/well in carbonate bicarbonate buffer, pH 9.5 was coated onto high affinity ELISA plates (Costar, Cambridge, MA) at 4°C over night. Wells were then blocked, respectively with 1% BSA or 5% non-fat dry milk in PBS, pH 7.4 for 2 h at 37°C and incubated with different dilutions of RSV. Wells were washed in PBS-Tween 20 (0.1%), pH 7.4 and sequentially incubated with goat anti-RSV polyclonal Ab at 90 μg/ml (Chemicon, Temecula, CA) and anti-goat IgG HRP conjugate (Sigma, St. Louis, MO). Wells were washed and developed with the TMB (substrate). Color development was stopped after 30 min by addition of 0.2 M sulfuric acid. The optical density was measured at 450 nm. For blocking experiments, RSV suspension was preincubated with either anti-RSV-G mAb (clone 131-2G specific to strain A_2, Chemicon, CA) or anti-RSV-F mAb (clone 133-1H specific to strain A_2, Chemicon, CA) at concentrations ranging from 20 to 100 μg/ml.

Production of recombinant RSV F protein and F-ICAM-1 interaction. To produce the recombinant F protein using baculovirus expression system, RSV F cDNA was amplified by RT-PCR as *Eco*RI and *Xba*I cassette (1.747 kb) and ligated at identical sites downstream to the polyhedrin promoter in the baculovirus vector pFastBacHTa (4.855 kb) (LifeTechnologies, Gaithersburg, MD). The gene sequence was confirmed through DNA sequencing. This vector also incorporated a six-histidine tag at the N-terminus of the RSV fusion protein F. Recombinant baculovirus having F glycoprotein was generated according to the manufacturer's instructions. Infection of Sf9 insect cells with this recombinant virus produced fusion protein F that was purified using Talon (Clontech, Palo Alto, CA) columns using manufacturer recommended procedures. To determine the binding of ICAM-1 to RSV F protein, microtiter plate was coated with either 800 ng of recombinant RSV F protein or ovalbumin as a control at 4°C overnight in carbonate-bicarbonate buffer, pH 9.5. Following washing, and blocking of the non-specific sites, varied concentrations of human soluble ICAM-1 was added and incubated at 37°C for 2 h. Following three washes, plates were incubated for 2 h with mouse anti-human ICAM-1 monoclonal antibody (2 μg/ml) (clone BBA4, R&D Systems, Minneapolis, MN). Bound antibody was detected by anti-mouse IgG peroxidase conjugate (1:1000 dilution) (Boehringer-Mannheim, Germany). Color was developed by addition of TMB substrate and optical density was read at 450 nm using an automated ELISA plate reader.

Statistical analysis. The difference between the treated and control cells was analyzed by Student's t test. Differences between groups were considered significant at P values less than 0.05.

RESULTS

RSV and ICAM-1 colocalize on plasma membrane of HEp-2 cells. HEp-2 cells, a transformed human epithelial cell line, commonly used to propagate RSV, was infected with RSV strain A_2, was assayed for ICAM-1 expression following RSV infection by flow cytometry. The uninfected HEp-2 cells showed constitutive expression (3.6%) of ICAM-1. Surface ICAM-1 expression increased by four- and eightfold, respectively, 24 and 48 h post RSV infection (data not shown). RSV antigen expression was detected both on the plasma membrane

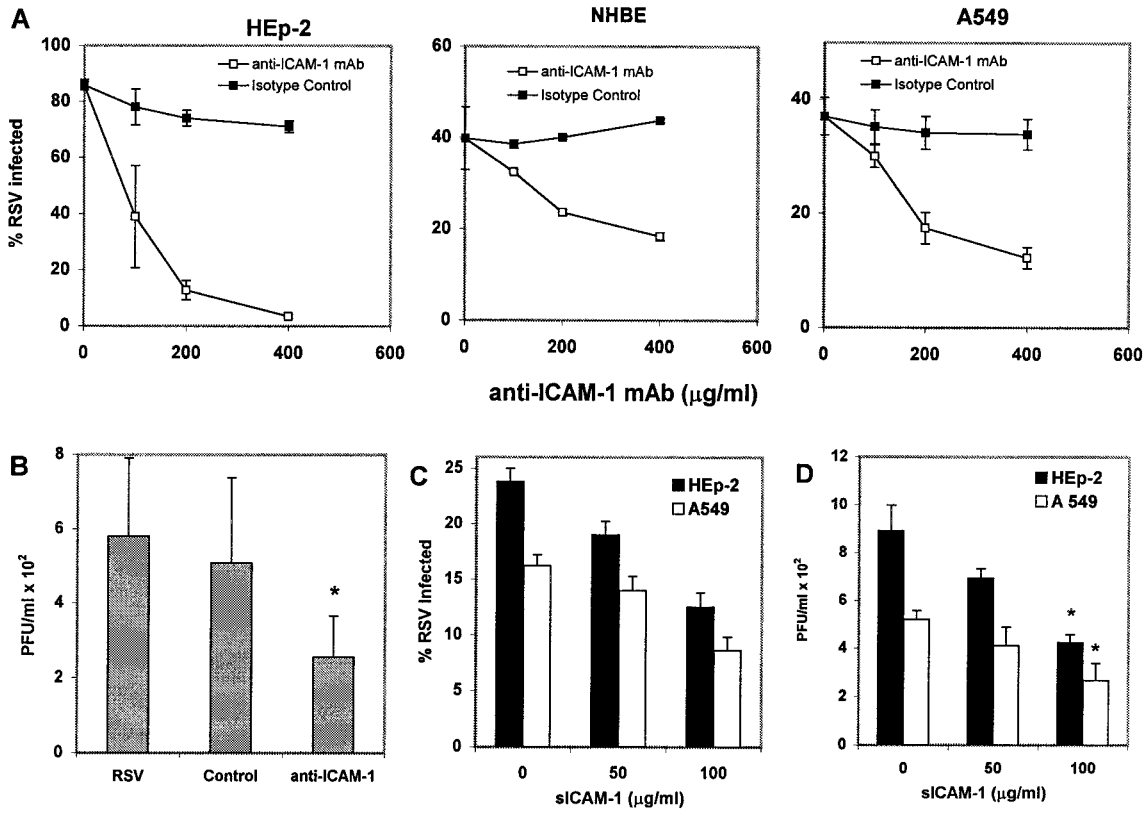

FIG. 2. The preincubation of epithelial cells with an anti-ICAM-1 mAb or RSV with sICAM-1 inhibits RSV infection. (A) Anti-ICAM-1 mAb treatment inhibited RSV infection of epithelial cells. The % of RSV-infected HEp-2, NHBE, and A549 cells after preincubation with anti-ICAM-1 mAb (□) or an isotype control Ab (■) was estimated as described. Each value represents mean ± SEM. The experiment was repeated for each cell line with similar results and a representative experiment for each of the cell lines is shown. (B) Preincubation of HEp-2 cells with anti-ICAM-1 mAb decreased the number of syncytia forming plaques. The cells were treated with 400 μg/ml of anti-ICAM-1 antibody, or an isotype matched control antibody or medium alone for 3 h and then infected with RSV. The syncytia forming plaques were enumerated at 72 h post infection. The experiment was repeated and a representative experiment is shown. Bar represents mean ± SD. $*P < 0.05$. (C and D) Preincubation of RSV with sICAM-1 decreased virus infection of cultured epithelial cells. RSV was preincubated for 30 min with various concentrations of sICAM-1 at room temperature, and subsequently used for infection of both HEp-2 and A549 cells. The percentage of RSV-infected cells was determined by immunofluorescence (C). Viral titer was determined by plaque assay at 72 h post infection (D). The experiment was repeated and a representative experiment is shown. Each bar represents mean ± SD. $*P < 0.05$. The preincubation of RSV with an unrelated protein, BSA, had no effect on the RSV infection (data not shown).

and in the cytoplasm of RSV infected cells (72 h pi), as determined by confocal microscopy, whereas ICAM-1 expression was localized mostly to the plasma membrane (Fig. 1). The colocalization of RSV and ICAM-1 on HEp-2 cell surfaces (Fig. 1D) suggested that RSV associates with ICAM-1 on epithelial cells.

Blocking of ICAM-1 significantly reduces RSV infection of epithelial cells. To examine if the blockage of ICAM-1 would inhibit the initiation of RSV infection, HEp-2 cells were infected with RSV after having been incubated with various concentrations of mAbs to human ICAM-1 (Fig. 2A). A dose-dependent reduction in the number of infected cells occurred with increasing concentrations of anti-ICAM-1 mAbs, as determined by immunofluorescence staining of infected cells. The greatest inhibition (96%) of RSV infection was observed with HEp-2 cells at 400 μg/ml of anti-ICAM-1 mAbs. In contrast, preincubation of cells with the same concentrations of isotype-matched IgG control showed no significant reduction in RSV infection.

To exclude the inhibition as a HEp-2 cell specific phenomenon, two other cell lines, A549 and NHBE, were examined for inhibition of RSV infection by anti-ICAM-1 mAbs. NHBE is a normal human primary cell line derived from bronchial explants, whereas A549 is a transformed lung type II alveolar epithelial cell line. NHBE and A549 exhibited a decrease in RSV infection by 54 and 67%, respectively, when preincubated with 400 μg of anti-ICAM-1 mAb/ml (Fig. 2A). The effect of blocking of ICAM-1 on HEp-2 cells on RSV replication was assessed by determining the number of syncytia forming plaques. The anti-ICAM-1 mAb treated and RSV infected cells showed 56% reduction in the number of plaques ($P < 0.01$) compared to the RSV infected cells (Fig. 2B), whereas no significant reduction was observed when cells were preincubated with the same

concentration of isotype matched IgG control. This result rules out the possibility that a large amount of IgG may cause network formation on the cell surface and may sterically block virus entry. To see the role of RSV binding to ICAM-1 on cell surface, the virus particles were precoated with excess sICAM-1 for 30 min and then used for infection. Viral titer decreased significantly when sICAM-1 preincubated viral inoculum was used for infection (Figs. 2C and 2D) in comparison to the control, where non-specific protein (BSA) preincubated viral sample was used for infection (data not shown). Highest reduction in viral titers of 52% ($P < 0.05$) and 49% ($P < 0.05$) in HEp-2 and A549 cells, respectively was observed with 100 μg/ml of sICAM-1 when compared to the 0 μg/ml. The viral titer was reduced to 22 and 20%, respectively, for HEp-2 and A549 cells at a concentration of 50 μg/ml. Similar reduction was observed when infected cells were counted by immunofluorescence (Fig. 2C). This further confirms that the binding of RSV to ICAM-1 on epithelial cell surface is important in RSV infection.

The intracellular viral replication was determined 24 h post infection by semiquantitative RT-PCR analysis of the RSV nucleoprotein (N) mRNA, to determine if the reduction of RSV infection was due to decreased viral replication leading to a decreased viral mRNA in the host cell. The preincubation of HEp-2 cells with anti-ICAM-1 mAb inhibited RSV mRNA levels in a dose-dependent manner, with the greatest inhibition (79% compared to control) observed at a concentration of 400 μg of anti-ICAM-1 mAb/ml medium (Fig. 3, upper panel). HEp-2 cells treated with anti-ICAM-1 mAbs failed to induce mRNA expression of the chemokine RANTES and the mediator ET-1, 24 h post-infection (Fig. 3, lower panel), which are normally induced in RSV infection (24, 25). These results indicate that ICAM-1 plays a key role in the RSV infection of epithelial cells and subsequent secretion of these inflammatory mediators.

RSV binds to immobilized or cellular ICAM-1 via its F protein. To determine if RSV can bind to ICAM-1 in the absence of cellular factors, the interaction of RSV with ICAM-1 was examined by an ELISA. Purified soluble ICAM-1 was applied to wells of ELISA plates and a dose dependent binding of RSV with ICAM-1 was observed (Fig. 4A). The lack of ICAM-1 binding to conditioned medium (in the absence of RSV) or that of RSV to an unrelated antigen, bovine serum albumin, indicated that the binding between ICAM-1 and RSV is specific (Fig.4A). To determine which of the RSV proteins interacts with ICAM-1, the RSV suspension was incubated with mAbs to either the F or G protein and the degree of inhibition of RSV binding to ICAM-1 was assayed. Incubation with mAbs to either the F or G protein decreased RSV-ICAM-1 binding by 80 and 36%, respectively (Fig. 4B). Simultaneous incubation of RSV

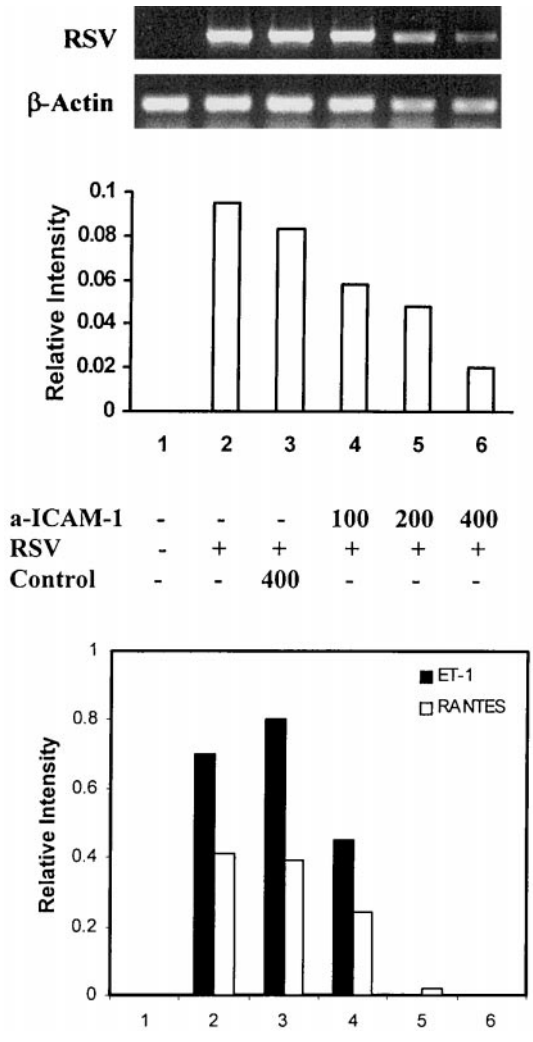

FIG. 3. The preincubation of HEp-2 cells with anti-ICAM-1 mAb inhibited the expression of the RSV N, RANTES and ET-1. The groups were as follows: uninfected cells (1), HEp-2 cells were treated with PBS (2), mouse IgG_1 antibody (control) 400 μg/ml (3), or with increasing concentration of the anti-ICAM-1 mAb at 100 μg/ml (4), 200 μg/ml (5), or 400 μg/ml (6) and subsequently infected with RSV. Total RNA was subjected to semiquantitative RT-PCR analysis using specific primers for the RSV N, RANTES, and ET-1 mRNAs and β-actin (as internal control). RT-PCR was done in triplicate and repeated three times and a representative band is shown. The products were quantified by densitometry and the band intensity for RSV N gene product (upper panel) and for RANTES and ET-1 (lower panel) normalized to β-actin, are plotted.

with blocking antibody (either to G or F) showed results identical to that of the prior incubation of RSV with the corresponding antibody (data not shown). To further confirm this, the purified recombinant F protein produced by a baculovirus expression system was found to bind to ICAM-1 in a dose-dependent fashion in a far-ELISA (Fig. 4C), whereas no binding was observed between ICAM-1 and immobilized ovalbumin. The baculovirus expressed recombinant F protein was able to cleave and fold correctly in insect cells and was

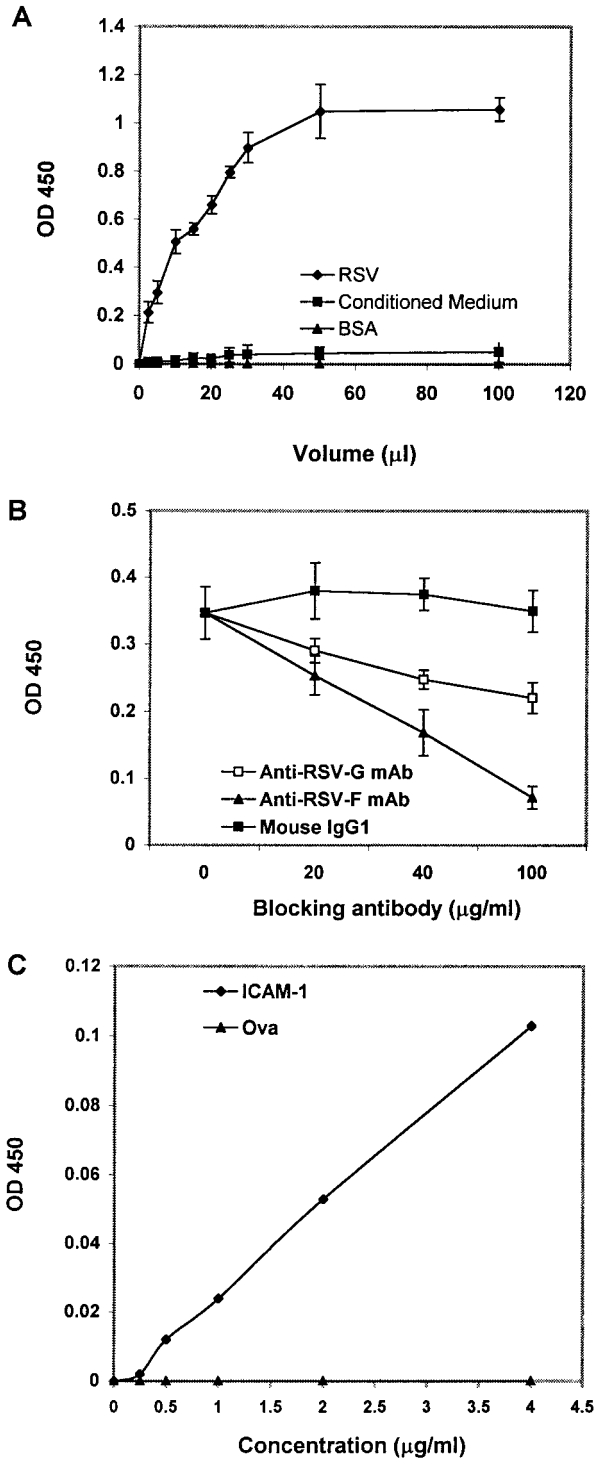

FIG. 4. Interaction of ICAM-1 with RSV in cell-free system. (A) RSV bound to immobilized ICAM-1 in a dose-dependent manner. Microtiter plates were coated with ICAM-1 and incubated with an increasing volume of RSV suspension (◆) or conditioned medium (■). Plates coated with BSA (control) were incubated with an increasing volume of RSV suspension (▲). Bound RSV was determined by ELISA. The optical density (mean ± SD) was plotted. The experiment was repeated three times with similar results and a representative experiment is shown. (B) Inhibition of RSV binding to ICAM-1 was examined by preincubation of RSV with mAbs to the G (□) or the F (▲) or mouse IgG_1 as control (■). (C) ICAM-1 binds to immobilized recombinant F protein. The recombinant F protein produced by the baculovirus system was coated on microtiter plates and incubated with an increasing concentration of ICAM-1 (◆), and the F protein bound to ICAM-1 was determined by ELISA. As a control increasing concentration of ICAM-1 was bound to ovalbumin-coated plates (▲). The optical density was plotted against the concentration of ICAM-1. The experiment was repeated with similar results and a representative experiment is shown.

immunologically active (data not shown). These results suggest that RSV can bind to ICAM-1 in the absence of other cellular factors and this binding is primarily via its F protein.

DISCUSSION

The evidence that ICAM-1 plays a significant role in RSV infection is based on the demonstrations that: (i) antibodies to ICAM-1 can significantly inhibit RSV infection in cultured epithelial cells; (ii) soluble form of ICAM-1 binds to RSV and significantly decreases RSV infection in epithelial cells; and (iii) RSV binds to purified ICAM-1 via its F protein; which plays an important role in the fusion process.

The blocking experiments using neutralizing anti-ICAM-1 mAbs provide the strongest evidence that ICAM-1 plays a role in RSV infection. These results show a significant inhibition of RSV infection in cultured epithelial cells, as determined by the percentage of infected cells (by immunofluorescence), by syncytia formation (plaque assay), and by a semiquantitative RT-PCR (RSV replication). An important finding is that preincubation with anti-ICAM-1 mAbs of three different epithelial cell lines, including both primary and transformed epithelial cells, inhibits both RSV infection and replication. Preincubation of all these cell lines with the same concentrations of isotype matched IgG control did not inhibit RSV infection, ruling out the possibility that the large amount of IgG may cause network formation on the cell surface and thus sterically block virus entry into the host cells. This also suggests that the inhibition of RSV infection is not due through nonspecific permutation. This indicates that ICAM-1 may provide a generalized mechanism for RSV infection in epithelial cells.

The amount of antibody needed to obtain maximal inhibition is relatively high. The reason for this is presently unclear. The requirement for a high concentration of antibodies for inhibition may be attributed to two mutually non-exclusive possibilities. First, ICAM-1, per se, appears to recycle between the cytoplasm and membrane. It was reported from the kinetic binding studies of a ^{125}I-labeled mAb to ICAM-1 that ~8% of membrane ICAM-1 in endothelial cells was internalized at a rate of ~3200 molecules/min (26). Further, this internalization pathway was directly de-

pendent on the level of ICAM-1 expression on the cell surface. Since the identical anti-ICAM-1 antibody was used in our studies, it is likely that a portion of these anti-ICAM-1 antibodies undergo internalization in epithelial cells due to a continuous recycling process. Providing this is the case, the difference in the magnitude of the inhibition of RSV infection in different cell lines may be linked to their level of ICAM-1 expression. Second, the anti-ICAM-1 blockade of membrane ICAM-1 does not interfere with the *de novo* synthesis of ICAM-1; rather it may enhance ICAM-1 synthesis. In support of this possibility, the cross-linking of ICAM-1 by the anti-ICAM-1 antibody has been reported to upregulate IL-1β expression in cells, which in turn, upregulates ICAM-1 expression (18). Also, our results and those of others indicate that RSV infection increases surface ICAM-1 expression on epithelial cells (27, 28). It is therefore not surprising that excess ICAM-1 antibodies are required to block not only the preexisting but also the newly synthesized ICAM-1. Irrespective of the dose of antibody required, the blocking of ICAM-1 by anti-ICAM-1 mAb inhibited RSV infection and consequently, some of the potent mediators released by these cells.

The importance of RSV binding to ICAM-1 on epithelial cell surface is further demonstrated by the decrease in viral titer when virus is preincubated with sICAM-1 prior to infection. The binding sites for ICAM-1 on the virus are thus blocked and are not available for the cell surface ICAM-1. sICAM-1 also inhibits infection of major rhinovirus subgroups for which ICAM-1 is known to be the cell surface receptor (20, 22). There was about 90% inhibition of rhinovirus cytopathic effect (CPE) with sICAM-1 (10 μg/ml) preincubation (29). A significant reduction of 52% ($P < 0.05$) and 49% ($P < 0.05$) using 100 μg/ml and 22 and 20% using 50 μg/ml in viral titer was observed by plaque assay in HEp-2 and A549 cells, respectively (Fig. 2C) with sICAM-1 preincubation. Preincubation of RSV with equal concentrations of BSA used as a control antigen had no effect on RSV plaque formation (data not shown). The extent of inhibition of viral infection observed in both cases cannot be directly compared, as the method of CPE determination for rhinovirus infection (29) is not comparable to plaque assay (PFU/ml) in the strict sense. In support of our data, very large amount of sICAM-1 (1 mg/ml) was required to inhibit the rhinovirus infection induced expression of ICAM-1 on epithelial cell surface (30). The specificity of virus inhibition indicates that sICAM-1 prevents infection not as a result of generalized effects on the cell's ability to support viral replication, but rather by inhibition of virion interaction with cell surface ICAM-1.

To understand the mechanism of ICAM-1 participation in RSV infection, the interaction of ICAM-1 with RSV was examined in absence of cellular factors. The dose-dependent binding of RSV to immobilized ICAM-1 and of immobilized recombinant F protein to ICAM-1 and a significant inhibition of the RSV-ICAM-1 interaction by anti-F antibodies, together suggests that the binding between RSV-ICAM-1 and F-ICAM-1 is specific. Use of immobilized glycoproteins, such as F protein and ICAM-1 in ELISA, is well documented in literature (16). Antibodies to the F protein have greater neutralizing ability than antibodies to the G protein (7, 8, 10–12). The evidence that the antibodies to F, but not to G, protein significantly inhibits RSV-ICAM-1 interaction (Fig. 4B) suggests that the RSV F-ICAM-1 binding may be a key event for the fusion of RSV into the host cell's membrane, which precedes RSV internalization. However, mechanism of such internalization is unclear. ICAM-1 has been reported to facilitate the internalization of a bound ligand, fibrinogen, in endothelial cells and in transfected COS cells (26). It is thus likely that ICAM-1 facilitates the internalization of RSV after the RSV F protein binds to ICAM-1 on the cell surface. Interestingly, it has been reported that the RSV-F protein also binds to RhoA, which is necessary for syncytia formation (16), although the mechanism underlying interaction between RhoA and RSV-F protein remains elusive.

The evidence that ICAM-1 plays a key role in the initiation of RSV infection raises the possibility of developing novel approaches to treat RSV infection. It should be noted that although our studies are confined to the RSV A_2 strain, RSV, as opposed to the rhinovirus, is considered serologically monotypic; i.e., antiserum to A_2 neutralizes heterologous strains in *in vitro* assays and in experimental animals (9, 31). Since the RSV F-ICAM-1 binding facilitates RSV infection of host cells, efforts to neutralize RSV by soluble ICAM-1 may prove to be useful. In rhinovirus infections, the administration of a truncated form of soluble ICAM-1, $tICAM\text{-}1_{453,}$ prevented rhinovirus infection in chimpanzees (32). The potential of developing anti-rhinovirus drugs that block ICAM-1 from binding to a canyon of the rhinovirus coat has also been suggested (33, 34). Furthermore, in a rat model of parainfluenza induced bronchiolitis it was shown that treatment with a blocking ICAM-1 antibody significantly reduced virus induced increases in BAL neutrophils and lymphocytes by 70% and infection induced methacholine hyperresponses (35). In summary, the results of this study underscore the importance of ICAM-1 in the early stage of RSV infection and establish the F-protein-ICAM-1 interaction as a target for developing novel therapeutics against RSV infection.

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