Cell-Specific Expression of RANTES, MCP-1, and MIP-1α by Lower Airway Epithelial Cells and Eosinophils Infected with Respiratory Syncytial Virus

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Respiratory syncytial virus (RSV) is the major cause of acute bronchiolitis in infancy, a syndrome characterized by wheezing, respiratory distress, and the pathologic findings of peribronchial mononuclear cell infiltration and release of inflammatory mediators by basophil and eosinophil leukocytes. Composition and activation of this cellular response are thought to rely on the discrete target cell selectivity of C-C chemokines. We demonstrate that infection in vitro of human epithelial cells of the lower respiratory tract by RSV induced dose- and time-dependent increases in mRNA and protein secretion for RANTES (regulated upon activation, normal T-cell expressed and presumably secreted), monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein-1α (MIP-1α). Production of MCP-1 and MIP-1α was selectively localized only in epithelial cells of the small airways and lung. Exposure of epithelial cells to gamma interferon (IFN-γ), in combination with RSV infection, induced a significant increase in RANTES production that was synergistic with respect to that obtained by RSV infection or IFN-γ treatment alone. Epithelial cell-derived chemokines exhibited a strong chemotactic activity for normal human blood eosinophils. Furthermore, eosinophils were susceptible to RSV and released RANTES and MIP-1α as a result of infection. Therefore, the inflammatory process in RSV-induced bronchiolitis appears to be triggered by the infection of epithelial cells and further amplified via mechanisms driven by IFN-γ and by the secretion of eosinophil chemokines.

Respiratory syncytial virus (RSV) is responsible for virtually all the cases of bronchiolitis experienced during the first 2 years of life and for the vast majority of lower respiratory tract infections associated with wheezing between the ages of 2 and 5 (62). In addition to the clinical and epidemiological relationship between bronchiolitis and asthma later in life (23, 50), the two diseases are linked by common histopathological features characterized by profound inflammation of the airway mucosa. Along this line, necrosis of the bronchial epithelium associated with peribronchial and perivascular mononuclear cell infiltration is considered a hallmark of RSV infection, both in humans (1, 14, 15) and in animal models (19, 20, 41, 42, 57). Moreover, the presence of cell-specific inflammatory mediators in nasopharyngeal secretions and in tracheobronchial aspirates of children with bronchiolitis suggests that RSV infection triggers the migration to the airways and local activation of eosinophil and basophil leukocytes (10, 16, 49, 61, 63).

The specific antiviral immune response appears to play a role in the pathogenesis of RSV-induced airway inflammation. Development of an RSV-specific immunoglobulin E (IgE) response in the airways of infected children has been in fact associated with the release of histamine (63) and of leukotriene C4 (61). Recent studies suggest that other mechanisms may also trigger and sustain lung inflammation following RSV infection. In this regard, eosinophil cationic protein has been demonstrated in nasopharyngeal secretions of children with various forms of RSV-related airway disease (10, 16). Levels of eosinophil cationic protein were significantly higher in subjects with clinically proven bronchiolitis than in children with upper respiratory infection or with pneumonia but without wheezing and were significantly correlated with the degree of hypoxia (16).

Much of the cellular response at sites of tissue inflammation is controlled by gradients of chemotactic factors that direct leukocyte transendothelial migration and movement through the extracellular matrix. The composition of this cellular response is dependent on the discrete target cell selectivity of these chemotactic molecules. Chemokines, a newly identified family of small chemotactic cytokines, regulate the migration and activation of leukocytes and therefore play a key role in inflammatory and infectious processes of the lung (11, 40). Members of the C−branch of the chemokine family such as RANTES (regulated upon activation, normal T-cell expressed and presumably secreted), macrophage inflammatory protein−1α (MIP-1α), and monocyte chemotactic protein-1 (MCP-1) are chemotactic and activator factors for monocytes, basophils, and eosinophils, with no activity on neutrophils (2, 3, 5, 13, 26, 44). With regard to human T lymphocytes, RANTES, MIP-1α, and MCP-1 have been shown to be specifically chemotactic for CD4+ T cells of the CD45RO+ memory phenotype (36, 46, 47). Among other potential important function of RANTES and MIP-1α in allergic and virus-induced inflammation are their capacity to stimulate surface IgE-surface or IgG4-positive B cells for enhanced IgE and IgG4 production (28) and to induce degranulation of natural killer cells and cytotoxic T cells (55, 56). Moreover, these chemokines are present in lung tissue and bronchial lavage fluid of asthmatic subjects (4, 58). Human epithelial cells appear to be a dominant source of
many C-C chemokines, including RANTES (54) and MCP-1 (51, 53). Respiratory epithelial cells are the primary and, as in the case of RSV, virtually the only cell target of viruses which enter the airways (21). Therefore, to identify the mechanisms that can regulate the development of airway mucosa inflammation in viral infection, we have investigated the production of C-C chemokines by RSV-infected respiratory epithelial cells. The studies demonstrate that in vitro infection by RSV of epithelial cells from the large bronchi, bronchioles, and lung type II cells results in the induction and secretion of a cell-specific pattern of C-C chemokines. In contrast to the widespread secretion of RANTES by the infected respiratory epithelium, production of both MCP-1 and MIP-1α after RSV infection appears to be selectively localized in epithelial cells from the small bronchioles and lung. Moreover, since constitutive or cytokine-inducible MIP-1α gene transcription and protein secretion by epithelial cells have not been previously reported, these findings suggest the existence of a novel and specific mechanism by which RSV may trigger inflammation in the lung. Along this line, we demonstrate here that the chemokines produced by RSV-infected epithelial cells have biological activity relevant to the pathogenesis of allergic inflammation, as they exhibit chemotaxis for blood eosinophils. Finally, we show that human blood eosinophils are susceptible to RSV and elaborate and secrete the chemokines RANTES and MIP-1α in response to infection. Therefore, the pathologic process of inflammation in RSV bronchiolitis, initially triggered by the infection of airway epithelial cells, may be further sustained and amplified via autocrine mechanisms driven by the secretion of eosinophil chemokines.

MATERIALS AND METHODS

Culture of epithelial cells. Cultures of normal human bronchial epithelial cells from the trachea and main bronchi (normal human bronchial epithelial [NHBE] cells) or from the bronchioles (small airway epithelial [SAE] cells) were initially established at Clonetics Corp. (San Diego, Calif.) from normal human tissue according to referenced procedures (32). SAE cells were positive for cytokeratin (19 and negative for alkaline phosphatase, a marker of lung type II epithelial cells. NHBE and SAE cells were grown in BEGM (Clonetics Corp.) containing human recombiant epidermal growth factor (0.5 ng/ml), hydrocortisone (0.5 μg/ml), bovine pituitary extract (30 μg/ml), biliary microparticle (0.1 mg/ml), insulin (5 μg/ml), and gentamycin (50 μg/ml), amphotericin B (50 ng/ml), and 1% bovine serum albumin (for SAE culture). Cells were used in the experiments at the fourth passage. A549 cells (American Type Culture Collection, Rockville, Md.) were grown as monolayer in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml (all from Gibco BRL, Grand Island, N.Y.). All cell lines were maintained at 37°C in 5% CO2.

Eosinophil purification. Heparinized venous blood was obtained from normal volunteers and sedimented with 6% dextran. The leukocyte-enriched buffy coats were overlaid onto Ficoll-Paque (Pharmacia, Piscataway, N.J.) and centrifuged for 20 min. The granulocyte-containing cell pellets were collected and washed twice with cold calcium- and magnesium-free Hanks buffered salt solution (Gibco BRL) as previously described (29). Erythrocytes were removed by hypotonic lysis. Eosinophils were negatively selected with anti-CD16 immune-magnetic beads to remove neutrophils, using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified eosinophils were then incubated mechanically for 1 h at 37°C, and then medium (0.16 ml/cm2) was added to the culture flask. The infection was continued for the indicated times in a 37°C incubator. Supernatant were collected at 3, 6, 12, 24, or 48 h, centrifuged, and stored at -70°C for subsequent measurement of chemokines by specific enzyme-linked immunosorbent assay (ELISA). Cells were harvested from the same tissue culture flasks, and total RNA was extracted for the determination of chemokine mRNA. To assess the requirement for viral replication in the generation of chemokines, epithelial cells were treated with UV-inactivated preparations of RSV. In other experiments, chemokine production was determined in supernatants of epithelial cells that were simultaneously infected with RSV at an MOI of 0.2 and treated with IFN-γ (100 U/ml; Boehringer Mannheim, Indianapolis, Ind.).

Production of RANTES and MIP-1α by human eosinophils. Purified eosinophils (2 × 106 cells/ml) were infected with RSV at an MOI of 10 or cultured with control medium (RPMI 1640 with 2% FCS) at 37°C in 5% CO2. After 16 h of incubation, the supernatants were collected and stored at -70°C until used. To determine total content of RANTES and MIP-1α, eosinophils (2 × 106 cells/ml) were infected with RSV at 0.1 MOI for 2 h at 37°C. Following infection, the cells were washed twice with 2% Triton X-100.

Immunofluorescence assay for viral infection. Infection of epithelial cells and eosinophils exposed to RSV was determined by indirect immunofluorescence microscopy with minor modifications of the method previously described (27). Briefly, acetone-fixed cytospin smears of control or RSV-infected epithelial cells and eosinophils were incubated for 45 min at 37°C with a monoclonal antibody (MAB) (clone B5) directed against RSV F-glycoprotein (Fg) (59) or with an irrelevant mouse MAB of the same isotype (DAKO Corp., Carpinteria, Calif.) followed by incubation for 45 min at 37°C with a fluorescein isothiocyanate-conjugated sheep F(ab’), IgG antibody (1:200) (DAKO). After extensive washing, slides were counterstained with Evans blue (Sigma Chemical Co., St. Louis, Mo.) and examined with a fluorescence microscope (Nikon Optiphot) equipped with a photomicrographic attachment (Nikon HFX-DX).

ELISA for chemokines. Levels of immunoreactive RANTES, MCP-1, and MIP-1α were determined by using a commercially available ELISA (R&D Systems Inc., Minneapolis, Minn.) as instructed by the manufacturer. Cell-free supernatants were tested in duplicates. The RANTES, MCP-1, and MIP-1α ELISAs are sensitive to 2.5, 5, and 2 pg/ml, respectively, and have an intra-assay coefficient of variation of <5% and interassay coefficient of variation of <10%, according to the manufacturer's instructions.

RT-PCR for measurement of chemokine mRNA. Total RNA was extracted from control or RSV-infected epithelial cells by the guanidinium thiocyanate method (8) with RNAzol B (BIOTECX, Houston, Tex.), and reverse transcription (RT)-PCR was performed with Gene RNA PCR kit components (Perkin Elmer-Cetus, Norwalk, Conn.). In brief, 1 μg total RNA was added to 10 μl of RT mixture (5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1 mM deoxyribonucleoside triphosphates, RNase inhibitor [1 U/μl], Moloney murine leukemia virus reverse transcriptase [2.5 U/μl]), 2.5 μM random hexamers, and 0.5 U of RNaseH, and incubated at 42°C for 15 min in 20 μl of RT mixture to produce a total RNA mixture containing 10 μl of RT mixture consisting of 2 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl, AmpliTaq DNA polymerase (2.5 U/100 μl), and 0.4 μM sense and antisense primers for human RANTES, MIP-1α, MCP-1, and β-actin was added to the RT product mixture. The reaction mixture was then incubated at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 2 min for a total of 28 cycles (MCP-1) or 35 cycles (RANTES, MIP-1α, and β-actin). To ascertain that the PCR products were analyzed during the linear increase of the product, the optimal cycle number of RANTES, MIP-1α, and MCP-1 PCR was determined by either using a constant amount of cDNA template or adding fivefold serial dilutions of the target cDNA to the PCR mixture. Sequences of PCR primers used have been previously reported (4). PCR products were resolved alongside pUC18HaeIII DNA marker (Sigma) on a 1.8% agarose gel containing ethidium bromide, visualized under UV light, and photographed.

Eosinophil chemotaxis. Eosinophil chemotaxis was studied by using 5-μm polycarbonate membranes in Boyden microchambers as previously described (3). Briefly, 30 μl of RSV-CM was placed in the lower compartment of the Boyden chamber and incubated for 3 h at 37°C in the presence of 100 μl of eosinophil suspension (105 cells) in the upper compartment. For neutralizing studies, anti-RANTES MAb (10 μg/ml; R&D Systems) or irrelevant isotype control MAb IgG (DAKO) was added to the RSV-CM in lower compartments. The concentration of the neutralizing antibody was based on the inhibition curve of biologic effect provided by the manufacturer (R&D Systems). Uninfected supernatant was used as a negative control for RSV-CM. In control activating factor (PAF; 10-8 M) was used as a positive control. At the end of incubation, the membranes were removed and stained with Wright's stain. Migrated eosinophils were counted in 10 random high-power fields. All slides were analyzed by two independent observers blinded to the experimental conditions. Each experiment was performed in duplicate.

Experimental design. Determination of RANTES, MCP-1, and MIP-1α protein release and mRNA expression in epithelial cells. Epithelial cells grown at 90% confluence were infected with RSV at an MOI of 1 or, in dose-response experiments, at MOIs of 0.2, 0.5, 1, and 5. To infect the cells, frozen pRSV stock was rapidly thawed and diluted with MEM containing 2% FBS (AS59) or with BGM (NHBE and SAE). The virus was added immediately to the flask (0.04 ml of diluted virus/cm2 of area) to remove the culture medium. In chemiluminescence experiments, an equivalent amount of sucrose-pretreated uninfected Hep-2 cells was added to epithelial cells. After addition of virus, the flask were rocked mechanically for 1 h at 37°C, and then medium (0.16 ml/cm2) was added to the culture flasks. The infection was continued for the indicated times in a 37°C incubator. Supernatant were collected at 3, 6, 12, 24, or 48 h, centrifuged, and stored at -70°C for subsequent measurement of chemokines by specific enzyme-linked immunosorbent assay (ELISA). Cells were harvested from the same tissue culture flasks, and total RNA was extracted for the determination of chemokine mRNA. To assess the requirement for viral replication in the generation of chemokines, epithelial cells were treated with UV-inactivated preparations of RSV. In other experiments, chemokine production was determined in supernatants of epithelial cells that were simultaneously infected with RSV at an MOI of 0.2 and treated with IFN-γ (100 U/ml; Boehringer Mannheim, Indianapolis, Ind.).
cells; ‡, P > 0.05 compared with uninfected control cells; †, P < 0.02 for A549 cells compared with SAE and NHBE cells; §, P < 0.01 for SAE cells compared with NHBE cells.

Statistical analysis. Analysis of data was performed with the aid of Sigma Stat software (Jandel Scientific, San Rafael, Calif.). The effects of viral infection and time on chemokine protein levels were analyzed by using the paired Student t test and Mann-Whitney rank sum test. For all analyses, P values of <0.05 were considered to be significant.

RESULTS

Infection of SAE cells by RSV. Human epithelial cells from the lower airways were used for these studies. NHBE and SAE cells were isolated from normal tissue of the large bronchi (two adult donors and two children of 6 and 8 years) and terminal bronchioles (two adults), respectively. A549, a cell line derived from an alveolar cell carcinoma of the lung, retains features of type II alveolar epithelial cells, including the ability to produce surfactant (33). While A549 and NHBE cells have been previously shown to be susceptible to RSV (17, 37), no information was available about the in vitro susceptibility of SAE cells to RSV. Thus, in initial experiments, monolayers of SAE cells were inoculated with RSV at an MOI of 1 for 48 h and the infection was determined by indirect immunofluorescence. Cytosin preparations of RSV-exposed SAE cells that were stained with a MAb directed against the Fgp of RSV showed typical intracytoplasmic fluorescent granular inclusions at 48 h postinfection (not shown). Time points later than 48 h were not analyzed since cells were almost completely detached from the plastic vessels as a consequence of massive damage. Similar patterns of positive fluorescence were found in preparations of NHBE cells and A549 cells exposed to RSV.

Bronchial, small airway, and lung type II epithelial cells infected with RSV secrete the C-C chemokines RANTES, MCP-1, and MIP-1α. Members of the C-C chemokine family such as RANTES, MCP-1, and MIP-1α are known to be potent chemoattractants and activators of T lymphocytes, monocytes, and eosinophils, all of which are present in inflammatory infiltrates or in respiratory secretions of RSV-infected children. Thus, we examined whether lower airway epithelial cells infected with RSV were induced to secrete these chemokines. Analysis of culture supernatant by ELISA showed that A549, NHBE, and SAE cells were able to express cell-specific patterns of C-C chemokines following RSV infection (Fig. 1). While RANTES and MIP-1α were virtually absent in 48-h supernatant of uninfected epithelial cells, MCP-1 was released in modest concentrations only by uninfected A549 cells. Infection with RSV induced the secretion of RANTES, MCP-1, and MIP-1α, although in terms of absolute levels, RANTES production predominated in all cell types. In particular, 48-h RSV-infected A549 cells produced significantly higher concentrations of RANTES (32,680 ± 11,328 pg/ml; mean ± standard deviation [SD]) than SAE cells (2,745 ± 440 pg/ml) or NHBE cells (1,223 ± 192 pg/ml) (P < 0.02). We found also a statistically significant difference between the concentrations of RANTES released by SAE cells compared with NHBE cells (P < 0.01). RSV infection failed to induce MCP-1 release by NHBE and SAE cells, but high levels of this chemokine were released by infected A549 cells (3,177 ± 1,261 pg/ml). Since previous attempts by others to demonstrate induction of MIP-1α in human epithelial cells treated with potent inflammatory cytokines (IL-1β, TNF-α, and IFN-γ) have been unsuccessful (7), it was to some extent unexpected to find that RSV strongly induced MIP-1α secretion by A549 (683 ± 227 pg/ml) and SAE (72 ± 39 pg/ml) cells but not by NHBE cells. The patterns and concentrations of chemokines in control or RSV-infected NHBE and SAE were comparable in all clones (i.e., donors).

Additional experiments were conducted to characterize the generation of these chemokines in relation to RSV titer and replication. To determine the effect of viral dose on RANTES generation, different MOIs were tested in cultures of NHBE and A549 cells (Fig. 2). Infection of epithelial cells with increasing doses of RSV induced a proportional increase in

![FIG. 1. C-C chemokine secretion by RSV-infected epithelial cells.](http://jvi.asm.org/)

NHBE, SAE, and A549 cells were infected with RSV at an MOI of 1 (RSV) or cultured in control medium (Ctrl) for 48 h. RANTES, MCP-1, and MIP-1α concentrations were determined in the supernatant by specific ELISA. The results are expressed as mean ± SD of five experiments. * P < 0.05 compared with uninfected control cells; †, P < 0.02 for A549 cells compared with SAE and NHBE cells; §, P < 0.01 for SAE cells compared with NHBE cells.

![FIG. 2. Effect of RSV infectious dose and UV-inactivated RSV on RANTES production by airway epithelial cells.](http://jvi.asm.org/)

NHBE and A549 cells were exposed to UV-inactivated RSV (UV). Data are expressed as mean ± SD of three experiments. * P < 0.05 compared with control or with UV-inactivated RSV.

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RANTES release. Furthermore, the requirement of infectious virus for RANTES production was confirmed by the failure of UV-inactivated RSV pools (MOI of 1) to induce detectable levels of RANTES in both NHBE and A549 cell cultures. The dose-response curves for MIP-1α and MCP-1 in RSV-infected A549 and SAE cells, respectively, were comparable to that of RANTES (not shown).

**Time course of chemokine secretion and mRNA expression after RSV infection.** For the time course experiments, aliquots of culture supernatants were assayed for immunoreactive RANTES and MIP-1α at different time points after RSV infection or after exposure of epithelial cell monolayers to control medium (Fig. 3). Starting at 12 h, a progressive increase in RANTES concentrations could be detected in supernatants of RSV-infected A549, SAE, and NHBE cells. On the other hand, RANTES levels in uninfected cells remained either undetectable or at low levels of detection throughout the 48-h culture. Later time points were not tested because of the extensive cell damage. MIP-1α secretion, tested in A549 and SAE cells, was characterized by a slower kinetics compared to RANTES. By 12 h, MIP-1α was still undetectable in control or infected cells. MIP-1α immunoreactivity, however, appeared in cell supernatant at the 24-h time point and peaked at 48 h in RSV-infected A549 and SAE cells while remaining undetectable in uninfected cell cultures.

Time-dependent changes in the steady-state levels of chemokine mRNA were assayed by RT-PCR in NHBE and A549 cells (Fig. 4). Uninfected cells expressed levels of RANTES mRNA close to the detection limit at all time points tested (6, 24, and 48 h for NHBE cells and 48 h for A549 cells). However, after RSV infection, expression of RANTES mRNA both in NHBE and A549 cells increased within 6 h and up to 48 h, a time at which cells lose viability. Analysis of MIP-1α and MCP-1 mRNA expression was also carried out in A549 cells. MIP-1α mRNA was not expressed in uninfected A549 cells but was strongly induced by RSV starting at 6 h postinfection. In agreement with protein levels (Fig. 1), we found constitutive expression of mRNA for MCP-1 in uninfected A549 cells and increased mRNA abundance at 6 to 24 h postinfection. Therefore, the secretion of RANTES, MCP-1, and MIP-1α protein in infected epithelial cells was paralleled by an increase in the levels of mRNA, suggesting that the induction of these chemokines by RSV was mediated through enhanced mRNA production and/or mRNA stabilization.

**FIG. 3.** Kinetics of RANTES and MIP-1α accumulation in supernatant from RSV-infected A549, SAE, and NHBE cells. Epithelial cells were infected with RSV at an MOI of 1 (RSV) or cultured in control medium (Ctrl). After indicated time of incubation, supernatants were collected for RANTES and MIP-1α determination by ELISA. The results are expressed as mean ± SD of four experiments. *P < 0.05 compared with control at each time point.

**FIG. 4.** Time-dependent expression of chemokine mRNA by RSV-infected NHBE and A549 cells. Total RNA was extracted from control (Ctrl) and RSV-infected (RSV) cells at the indicated time. RT-PCR was performed with specific primers for human RANTES, MIP-1α, MCP-1, and β-actin. PCR products were visualized with ethidium bromide staining on agarose gel. Expression of the housekeeping gene encoding β-actin is shown for comparison to demonstrate relative equal loading of the RT-PCR mixtures for all samples. A representative experiment from three performed on separate cell culture samples is shown.
Synergistic effect of RSV and IFN-γ on RANTES production by epithelial cells. RSV infection has been shown to induce IFN activity in vivo (22) and in vitro which modulates the expression of immunoregulatory molecules on respiratory epithelial cells (17). Therefore, we decided to investigate in our model of chemokine secretion the concurrent effect of RSV and IFN-γ, a combination likely present in the airway mucosa of infected infants. NHBE cells were either infected with RSV (MOI of 0.2), treated with IFN-γ, or infected with RSV and treated with IFN-γ simultaneously. Supernatant was analyzed at 48 h for the presence of immunoreactive RANTES. Since 10-fold increments of IFN-γ (1, 10, and 100 ng/ml) have been shown previously to induce the production of comparable amounts of RANTES by bronchial epithelial cells (54), a single concentration of IFN-γ (100 U/ml [5 ng/ml]) was chosen in our experiments. As shown in Fig. 5, treatment of NHBE cells either with RSV at a low infectious dose or with IFN-γ induced the production of RANTES (164 ± 13 or 390 ± 61 pg/ml, respectively). Coincubation with RSV and IFN-γ induced strong upregulation of RANTES secretion (810 ± 89 pg/ml) that was synergistic with respect to that obtained by RSV infection or IFN-γ treatment alone.

Bioactivity of RSV-CM and contribution of epithelial cell-derived RANTES to eosinophil chemotaxis. The bioactivity of RSV-infected airway epithelial cell supernatant was investigated in the context of eosinophilic inflammation. For this purpose, eosinophils isolated from the blood of normal human donors were tested in Boyden microchambers for their chemotactic response to RSV-CM generated by infected NHBE or A549 cells. PAF (10⁻⁷ M), a major eosinophil chemoattractant, was used as a positive control (3). In four separate experiments, RSV-CM from NHBE and A549 cells (Fig. 6) induced 7- and 5.5-fold increases, respectively, in the number of migrated eosinophils compared to eosinophil migration in the presence of medium from uninfected epithelial cells. The number of eosinophils that migrated in response to NHBE RSV-CM (84.3 ± 13.8; mean ± SD) or in response to A549 RSV-CM (115 ± 24.4) was similar to the number of eosinophils that migrated in response to PAF (83.5 ± 10.6 and 76.2 ± 8.8). Since human RANTES has been previously shown to induce in vitro chemotaxis of human blood eosinophils (3), we determined the contribution of RANTES to the overall eosinophil chemotactic activity secreted by RSV-infected NHBE and A549 cells. Addition of neutralizing anti-RANTES MAb in concentration (10 μg/ml) sufficient to neutralize nearly 100% of the chemotactic activity of 1 μg of recombinant human RANTES per ml significantly reduced by approximately 33 and 51% the eosinophil chemotaxis induced by NHBE and A549 RSV-CM, respectively (P < 0.01).

RSV-infected eosinophils produce and secrete RANTES and MIP-1α. Following the process of transendothelial migration into the airway mucosa, the contribution of eosinophils to the inflammatory response may reflect their ability to release chemokines (38). However, factors that regulate the production and secretion of chemokines by eosinophil are not known. For this reason, we examined whether human eosinophils exposed to RSV were induced to express and secrete C-C chemokines. To determine if RSV was able to infect eosinophils, we performed studies using a well-established immunofluorescence assay previously used for the determination of infection in epithelial cells and macrophage (9, 27). Human eosinophils, isolated from the blood of normal individuals to a purity >99%, were cultured with control medium or were exposed to RSV. After 2 and 16 h, eosinophils were then stained with a MAb against RSV Fgp or with an isotype control. Cytospin preparations of eosinophils that were cultured in medium without virus were clearly negative (Fig. 7A), while those of eosinophils

![Graph](https://via.placeholder.com/150)

**FIG. 6.** Biological activity of RANTES released by RSV-infected NHBE and A549 cells. Eosinophil chemotaxis was determined in the presence of supernatant from RSV-infected cells (RSV-CM) or from uninfected cells (Ctrl). Neutralizing experiments were performed in the presence of specific anti-RANTES MAb or mouse IgG isotype control. PAF (10⁻⁷ M) was used as a positive control. Results are expressed as mean ± SD of four determinations for each set of experiments. *, P < 0.01 for RSV-CM, RSV-CM plus mouse IgG, and PAF compared with control; **, P < 0.01 for RSV-CM plus anti-RANTES MAb compared with RSV-CM.
exposed to RSV for 2 h show an intense immunofluorescence staining, concentrated in a pericytoplasmic halo (Fig. 7B). After 16 h of exposure to RSV, typical RSV intracytoplasmic granular fluorescence immunoreactivity, identical to that one previously shown in epithelial cells, was observed (Fig. 7C). Eosinophils that were stained with an isotype MAb were consistently negative (not shown).

To determine if eosinophils were able to elaborate the two C-C chemokines RANTES and MIP-1α in response to RSV infection, freshly isolated eosinophils were cultured in medium or were infected with RSV for 16 h. Total cellular content of RANTES and MIP-1α in uninfected eosinophils was also determined in cell lysates obtained by Triton X-100. The cell supernatant was then collected for the determination of RANTES and MIP-1α immunoreactivity by ELISA. As shown in Fig. 8, uninfected eosinophils spontaneously released RANTES and MIP-1α in concentrations close to the low level of detection (8.6 ± 3.5 and 4.2 ± 2.1 pg/ml, respectively; mean ± SD). Total content of performed RANTES in cell lysates was found in the range of 151 ± 126 pg/ml, while total MIP-1α remained at the low level of detection (4.2 ± 0.8 pg/ml). On the other hand, eosinophils that were exposed to RSV for 16 h released in the culture supernatant a significant amount of both RANTES (1,585 ± 623 pg/ml) and MIP-1α (133.8 ± 79.6 pg/ml). These results suggest that newly synthesized rather than a stored form of RANTES and MIP-1α were secreted by the RSV-infected eosinophils.

**DISCUSSION**

RSV, the single most important respiratory pathogen in infancy and early childhood, stimulated the secretion of the proinflammatory C-C chemokines RANTES, MCP-1, and MIP-1α from epithelial cells of the human lower respiratory tract. Increased chemokine secretion was paralleled by increased steady-state mRNA levels, and it was strictly dependent on viral replication and infectious dose. The profile and concentrations of the C-C chemokines produced by the respiratory epithelium were characteristically cell specific. RSV infection induced the release of RANTES by epithelial cells isolated from all the segments of the lower respiratory tract examined in this study, i.e., major bronchi, bronchioles, and lung, and, as we previously reported, from the upper respiratory airways (45). With regard to the protein concentration, RSV-infected epithelial cells obtained from the distal portion of the bronchial tree (SAE) and type II cells (A549) produced significantly greater levels of RANTES than those from the major bronchi and the upper airways. cis-acting elements have been identified in the human RANTES promoter containing consensus binding sites for the nuclear factor (NF)-IL6 and NF-κB transcriptional activators (39). Our recent observations that RSV infection induces the activation of NF-IL6 (25) and NF-κB (18) in epithelial cells indicates that these transcription factors may be involved in the viral activation of RANTES in the airway mucosa. Studies addressing this possibility are in progress.

In contrast to the widespread expression of RANTES in the infected respiratory epithelium, RSV induced the release of MCP-1 only from A549 cells. The production of MCP-1 by A549 cells in response to TNF-α and IL-1β has been reported previously (53). In the studies presented herein, the relatively modest increase of RSV-induced MCP-1 mRNA, in comparison to the amount of protein released, suggests that expression of MCP-1 gene in epithelial cells may be controlled at several levels, including transcriptional and posttranscriptional. Although results generated with the use of a cell line to mimic primary cell cultures must be interpreted with caution, A549 cells have proven to be excellent models of pulmonary type II epithelial cells for studying the production of several bioactive factors and the mechanisms of chemokine gene regulation (18, 25, 34, 52). Moreover, we demonstrate for the first time that human epithelial cells, both A549 cells and the normal SAE cells, are able to express and secrete MIP-1α. Indeed, recent attempts by other laboratories to demonstrate cytokine-induced expression of MIP-1α in normal bronchial epithelial cells (7) and RSV-induced expression MIP-1β, a chemokine...
closely related to MIP-1α, by a transformed bronchial cell line (6) have been negative. These findings are consistent with our results demonstrating that following RSV infection NHBE cells release neither MCP-1 nor MIP-1α. Thus, the release of MCP-1 and MIP-1α by the airway epithelial cells appears to be regionalized in the distal segments of the bronchial tree and in the lung, where RSV-mediated necrosis of the epithelium and peribronchial cellular infiltration are associated with greater physiological changes, particularly in infants (24).

The combination of IFN-γ with a low dose of infectious virus induced a significant increase in RANTES production that was synergistic with respect to that obtained by RSV infection or IFN-γ treatment alone. Recent investigations in a BALB/c mouse model have demonstrated that the acute RSV infection was characterized by a Th-1-like response with increased IFN-γ production, decreased IL-4 and IL-5 production, pulmonary eosinophilic inflammation, and airway hyperresponsiveness (48). Eosinophil chemotaxis is usually observed in the context of a Th-2-type cytokine response and not a Th-1-type response. Therefore, our findings suggest the possibility of a novel mechanism, Th-2 independent, mediated by epithelial C-C chemokines by which IFN-γ can promote the migration of eosinophils to the lung and airway hyperresponsiveness following acute RSV infection.

To confirm the biologic activity of epithelial cell-derived chemokines, we show in these studies that supernatant from cells infected with RSV exhibited a strong chemotactic activity for eosinophils. Since eosinophil chemotactic activity in vitro is a known property of RANTES (3, 26), the presence of a neutralizing anti-RANTES MAb significantly reduced eosinophil chemotaxis induced by NHBE and A549 RSV-CM. Although the concentration of RANTES in RSV-CM from A549 cells was approximately thirty times higher than the concentration in RSV-CM from NHBE cells, we found that the rate of eosinophil chemotaxis in response to the two conditioned media did not differ substantially. In this regard, we have previously shown that concentrations of RANTES in the range of those present in NHBE RSV-CM (10^−10 M) and in A549 RSV-CM (10^−9 M) exhibit comparable chemotactic activities for human eosinophils (3). Furthermore, the finding that eosinophil chemotactic activity in RSV-CM was only partially inhibited by anti-RANTES MAb supports the notion that other chemokines known to be secreted by the infected epithelium, including MIP-1α and IL-8 (18), have a direct eosinophil chemotactic activity or are able to enhance chemotactic response to other agents (43). On the other hand, the primary goal of our studies was not to identify the contribution of each chemokine to the total eosinophil chemotactic activity present in the RSV-infected epithelial cell supernatant but rather to demonstrate the potential relevance of this phenomenon in the generation of eosinophilic inflammation.

We have previously shown that RSV is capable of direct stimulatory interaction with eosinophils, as indicated by the release of superoxide and leukotriene C4 (30) and by demonstration of piecemeal degradation by electron microscopy studies (29). In addition to the release of products such as oxygen radicals, eicosanoids, and cytoplasmic granule proteins, eosinophils can promote inflammation by virtue of their ability to elaborate proinflammatory cytokines and chemokines (38). However, factors that regulate the production and secretion of chemokines by eosinophils are not known. In this present study, we demonstrate that human blood eosinophils were susceptible to RSV and were able to elaborate and secrete the chemokines RANTES and MIP-1α following infection. Others have shown by in situ hybridization and Northern blot analysis that blood eosinophils from hypereosinophilic individuals express MIP-1α mRNA, but the production of MIP-1α protein was not tested in that study (12). Recently, both RANTES mRNA and protein have been demonstrated in eosinophils infiltrating late-phase cutaneous reactions after intradermal allergen challenge and in peripheral blood eosinophils isolated from atopic subjects with eosinophilia (35) or from normal donors (64). RANTES protein appeared to be stored within the eosinophils in association with cytoplasmic granules and to be released only after lysis of the cells (35). Thus, no experimental evidence to date indicates that inflammatory cytokines or other exogenous stimuli can regulate RANTES or MIP-1α gene transcription or protein synthesis and secretion in human eosinophils. Since the amount of RANTES and MIP-1α released by eosinophils after RSV infection was much higher than that observed in cell lysates, our studies demonstrate that production and release of these chemokines is indeed inducible in RSV-infected eosinophils. As a consequence of the eosinophil infection by RSV, the inflammatory response, initiated by airway epithelial cell chemokines, may be further sustained and amplified via autocrine mechanisms driven by the release of eosinophil chemokines.

The studies presented here suggest that the pattern, absolute levels, and relative concentrations, together with the kinetics of release of epithelial cell chemokines in different areas of the respiratory tract, are all factors that may significantly contribute to the various histologic and inflammatory features of RSV-induced airway disease. Selective production of MIP-1α and MCP-1 and the severalfold-higher amount of RANTES released by epithelial cells of the small bronchioles and lung in comparison to bronchial or upper airway epithelium, along with autocrine and paracrine mechanisms of amplification mediated by eosinophils and IFN-γ, may largely explain the findings of mononuclear cell infiltration and eosinophil and basophil migration and activation in RSV-induced bronchiolitis. Future studies, both in children with different forms of RSV-induced airway disease and in animal models, are needed to correlate the different parameters of histopathology, inflammation, and immune response with the expression and secretion of C-C chemokines in airway mucosa.

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