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Respiratory Syncytial Virus Stimulates Neutrophil Degranulation and Chemokine Release

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Neutrophil infiltration of the airways is a common finding in respiratory syncytial virus (RSV) bronchiolitis. Neutrophil-derived chemokines and neutrophil granule contents can cause further inflammation, hyperresponsiveness, and damage of the airways. In this study, peripheral blood neutrophils incubated with RSV (multiplicity of infection (MOI) = 10) induced IL-8, macrophage inflammatory protein (MIP)-1α, MIP-1β, and myeloperoxidase (MPO) release. In contrast, LPS induced only chemokine but not MPO release. RSV-induced chemokine and MPO release was nontoxic as assessed by trypan blue exclusion. The mechanism of RSV-induced chemokine release was shown to be transcription dependent since cytokine mRNA synthesis was not inhibited by dex. We conclude that the release of chemokines (IL-8, MIP-1α, MIP-1β) and granule enzymes (MPO) by RSV-stimulated neutrophils may contribute to the pulmonary pathology in RSV bronchiolitis. These in vitro findings showing that dex failed to consistently inhibit all the RSV-induced release of neutrophil inflammatory mediators may explain the variable efficacy of corticosteroids in the treatment of RSV bronchiolitis. The Journal of Immunology, 1999, 163: 2816–2820.

Respiratory syncytial virus (RSV) bronchiolitis is a major cause of wheezing in children less than 2 yr of age. It is also a culprit of 4500 deaths and 90,000 hospitalizations in the United States annually (2). Airway inflammation in RSV bronchiolitis is undoubtedly a multicellular process in which epithelial cells, eosinophils, and neutrophils are involved. Multiple cytokines have been shown to be released by RSV-stimulated epithelial cells such as IL-6, IL-8, IL-11, GM-CSF, macrophage inflammatory protein (MIP)-1α, and RANTES (3, 4). Epithelial cells have always thought of as major contributors in the inflammatory process of the airways during RSV infection.

However, within the last decade, neutrophils and their products have been demonstrated in the airways of patients and animal models with RSV infection and bronchiolitis (5–7), viral-induced asthma (8, 9), and sudden onset fatal asthma (10). Consequently, neutrophils are believed to be important in the inflammatory process of RSV bronchiolitis and acute asthma. Neutrophils can release multiple cytokines such as IL-1β, IL-6, IL-8, IL-11, TNF-α, TNF-γ, MIP-1α, and MIP-1β, among which, IL-1β, IL-6 (11), and IL-8 have been shown to be released from RSV-stimulated neutrophils.

Therapeutically, although corticosteroids are the most effective agents for airway inflammation in asthma, they have not been consistently effective in the treatment of RSV bronchiolitis (12). It has been shown that dexamethasone (dex) inhibits histamine and leukotriene B4 release from basophils (13, 14) and IL-8 release from LPS-stimulated neutrophils (15). However, the effects of corticosteroids on RSV-stimulated neutrophil activation have not been evaluated.

In this study, we demonstrated that RSV stimulated IL-8, MIP-1α, and MIP-1β release from neutrophils, as well as neutrophil degranulation. To explain the ineffectiveness of corticosteroid treatment in RSV bronchiolitis, we evaluated the effects of dex on the above cytokine release and neutrophil degranulation as determined by myeloperoxidase (MPO) release.

Materials and Methods

Reagents

The following reagents were purchased from the indicated sources: HEPES, lymphocyte separation media, glutamine, dex, actinomycin-D, polymyxin B (Sigma, St. Louis, MO), TRizol (Life Technologies, Gaithersburg, MD), [32P]UTP (Amersham, Arlington Heights, IL), FCS, RPMI 1640 (Life Technologies, Grand Island, NY).

RSV preparation

The Long strain of RSV (A2) was propagated in HEp-2 cells (American Type Culture Collection (ATCC), Manassas, VA; CCL 23) with RPMI 1640 medium supplemented with 10% FCS and antibiotics. When the HEp-2 cells showed cytopathology, supernatants were centrifuged through 30% glycerol in DMEM at 13,500 × g for 6 h at 4°C. The virus was then recovered by centrifugation at 11,000 × g for 6 h at 4°C to pellet the virus. The RSV was resuspended in buffer containing 100 mM MgSO₄, 50 mM HEPES, and 150 mM NaCl, and aliquots were stored in liquid nitrogen until used for experiments. RSV titers were determined on HEp-2 cultures under nutrient agarose and expressed as PFU per milliliter. For all experiments, a new vial of RSV was rapidly thawed and used immediately.

Heat inactivation (H-RSV) of the virus was accomplished by incubation at 65°C for 45 min; UV inactivation (UV-RSV) was conducted in a Stratagene (La Jolla, CA) UV-stratalinker apparatus using 1800 mJ of UV radiation. A mock RSV stock (M-RSV) was prepared in exactly the same
manner as the RSV preparation, with the exception that the HEp-2 cells were not infected with RSV.

**Neutrophil isolation**

Neutrophils were isolated from the venous blood of healthy adult volunteers by centrifugation on lymphocyte separation media, as described previously (16). The cell preparations routinely contained >98% neutrophils, with <1 platelet/100 neutrophils as determined by direct microscopic counting.

**Stimulation of neutrophils**

Neutrophils (10^7) were stimulated with either RSV (10 MOI), UV-RSV, H-RSV, M-RSV (with the same volume as RSV), or 100 ng/ml LPS and incubated in 1 ml of RPMI 1640 supplemented with 5% FCS and 2 mM t-glutamine at 37°C in a 5% CO2 incubator. After 18 h, the cell suspensions were centrifuged, and the supernatants were analyzed by ELISA for chemokines and MPO. Cell pellets were resuspended in normal saline and assessed for viability with the trypan blue exclusion method.

**Assays**

IL-8, MIP-1α, MIP-1β, and MPO were assessed by commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Analysis of mRNA**

All solutions used for isolation of RNA were treated with 0.1% diethyl pyrocarbonate and sterilized or prepared with diethyl pyrocarbonate-gloves and were autoclaved at 240°C on dry cycle.

Neutrophils (3 × 10^6 cells/ml) were isolated with or without RSV (10 MOI) in RPMI 1640 containing 5% FCS, 100 μg/ml each of penicillin and streptomycin, 2 mM t-glutamine, and 10 mM HEPES for the indicated times at 37°C in 14 ml polypropylene round-bottom tubes in an oscillating water bath. The incubations were terminated by centrifugation at 400 × g for 10 min at 4°C, and the cells were washed once with PBS at 4°C. Total RNA was isolated with TRIzol according to the manufacturer’s directions. The cell pellet was run through a 22-gauge needle five to six times and placed into a new 14-ml polypropylene round-bottom tube. Chloroform (200 μl/1 ml of TRIzol) was added, and the samples were mixed vigorously and incubated at room temperature for 2–3 min. The aqueous phase was collected after centrifugation at 11,000 × g for 15 min at 4°C. The RNA was precipitated with equal volumes of isopropyl alcohol at −70°C for 1 h.

The ribonuclease protection assay (RPA) was performed using a commercially available kit from PharMingen (San Diego, CA). In brief, the multiprobe (h-CK-5), which included templates for the chemokines IL-8, MIP-1α, and MIP-1β, as well as house keeping gene GAPDH, was labeled with [32P]UTP using T7 RNA polymerase. The labeled probe (6 × 10^5 cpm) was hybridized to 4 μg of total RNA for 16 h at 56°C. The ds mRNA probe hybrids were treated with RNase, followed by phenol/chloroform extraction. Protected hybrids were resolved on a 6% denaturing polyacrylamide sequencing gel, and, after drying, the gel was exposed to Kodak (Rochester, NY) Biomax film overnight at 70°C. A plot of the mobility of the probes vs the nucleotide length was used to predict the migration of the protected probe fragments.

**Statistical analysis**

Data were analyzed on a Macintosh computer using the Statview statistical software. Data are expressed as mean ± SEM. The significance of the difference between the test and the control groups was analyzed using either Student’s t test or ANOVA.

**Results**

**RSV-stimulated chemokine release from neutrophils is noncytotoxic**

Konig et al. demonstrated IL-8 release from RSV-stimulated neutrophils (17). However, it has not been determined whether RSV-stimulated cytokine release is via a noncytotoxic mechanism. We used trypan blue staining to determine whether overnight incubation with RSV was cytotoxic to neutrophils. In addition to IL-8, neutrophils also express and secrete the CC-chemokines MIP-1α and MIP-1β upon activation with LPS (18, 19). We stimulated neutrophils with RSV (10 MOI) or LPS (100 ng/ml) for 18 h at 37°C and assayed the cell culture supernatant for MIP-1α, MIP-1β, and IL-8 release. We found that, in addition to IL-8 release, RSV also induced MIP-1α and MIP-1β release. Figure 1A shows that RSV (10 MOI) induced significant (p < 0.05) MIP-1α (1153.4 ± 384 pg/ml) and MIP-1β (2941 ± 1213 pg/ml) release when compared with unstimulated neutrophils. RSV induced approximately twice as much MIP-1α and MIP-1β release as LPS. However, the difference was not statistically significant. Figure 1B shows that both RSV and LPS stimulated chemokine release in a noncytotoxic manner.

To determine whether contamination by LPS contributed to the stimulation of chemokine release by RSV, we added polymyxin B (20 μg/ml) to the culture media. In six experiments, neutrophil chemokine release induced by RSV was not affected by the addition of polymyxin B. RSV-stimulated IL-8 release was 2459 ± 60 pg/ml and 2549 ± 227 pg/ml with and without polymyxin B respectively. In the same experiments, IL-8 stimulation by LPS was completely suppressed by polymyxin B (483 ± 160 and 30 ± 21 pg/ml with and without polymyxin B, respectively). Similar results were also seen for MIP-1α and MIP-1β release (data not shown).

**RSV stimulates neutrophil degranulation**

We used MPO as a marker for degranulation of the neutrophil primary granules. Figure 2 shows that, after 18 h of stimulation,
RSV induced 147 ± 29 ng/ml of MPO release compared with an unstimulated release of 14 ± 2 pg/ml (p < 0.05). Although LPS induced neutrophil chemokine release, it did not stimulate MPO release. As with chemokine release, RSV-stimulated MPO release was also noncytotoxic (data not shown). Cytochalasin-B did not enhance RSV- or LPS-stimulated MPO release (data not shown).

To determine whether HEp-2 cell components from the RSV preparations might stimulate neutrophil degranulation, we compared M-RSV with RSV for stimulation of MPO release in three additional experiments. RSV stimulated MPO release (211 ± 38 ng/ml) whereas M-RSV did not significantly stimulate MPO release above unstimulated neutrophils (86 ± 34 and 63 ± 10 ng/ml, respectively). In addition, M-RSV did not stimulate release of MIP-1α, MIP-1β, or IL-8.

Viable RSV is not required for stimulation of neutrophil degranulation but is required for stimulation of chemokine release

To determine whether viable RSV is necessary for stimulation of neutrophil degranulation and chemokine release, we stimulated neutrophils with RSV, UV-RSV, and H-RSV and measured MPO and chemokine levels in the culture supernatants. Fig. 3A shows that inactivation of RSV with UV radiation did not significantly abate neutrophil MPO release, as compared with viable RSV, but inactivation with heat decreased MPO release by 32% (p < 0.05).

Konig et al. have shown that viable RSV is not necessary for induction of neutrophil IL-8 release (17). However, we found that IL-8, MIP-1α, and MIP-1β release markedly diminished when neutrophils were stimulated with UV-RSV or H-RSV, as compared with viable RSV (p < 0.05). Fig. 3B shows that UV or heat inactivation decrease the release of all three chemokines by more than 80%.

Dex inhibits RSV-stimulated chemokine release but not neutrophil degranulation

To determine whether the corticosteroid dex inhibits RSV-induced neutrophil activation, we assessed the effects of dex on chemokine and MPO release. Fig. 4 shows that IL-8 and MIP-1α release was significantly inhibited by 87% (p = 0.03) and 45% (p = 0.05), respectively, by 1 µM dex. Dex decreased RSV-stimulated MIP-1β release from 2941 ± 1213 pg/ml to 1589 ± 811 pg/ml. However, the difference was not statistically significant due to a wide range of MIP-1β release from the neutrophils of the five donors tested. Dex did not significantly inhibit MPO release in eight experiments. The amount of RSV-stimulated MPO release was 127 ± 15.3 ng/ml, compared with 108.8 ± 6.4 ng/ml when dex was added.

Delayed dex addition inhibits chemokine release

To determine whether chemokine release would still be suppressed when dex was added after the addition of RSV, we added dex at different time points ranging from 1 h before to 4 h after RSV stimulation. Fig. 5 shows that dex significantly inhibited IL-8 and MIP-1α release when added at all the time points indicated, with

![FIGURE 2. Determination of MPO release by RSV stimulation. MPO release from RSV- or LPS-stimulated neutrophils was assessed by ELISA. The data from four experiments are shown as mean ± SEM. *p < 0.05, compared among the indicated groups.](http://www.jimmunol.org/)

![FIGURE 3. Comparison of viable RSV and inactivated RSV on stimulation of neutrophil degranulation and chemokine release. MPO, MIP-1α, MIP-1β, and IL-8 levels were assessed by ELISA after 18 h of incubation neutrophils with RSV, UV-RSV, or H-RSV. The data from six experiments are shown as mean ± SEM. A, MPO release. * p < 0.05, compared with unstimulated neutrophils. +, p < 0.05, compared between the indicated groups. B, Chemokine release. * p < 0.05, compared with corresponding values from unstimulated neutrophils.](http://www.jimmunol.org/)

![FIGURE 4. Dex inhibition of IL-8 and MIP-1α release from RSV-stimulated neutrophils. Neutrophils were preincubated with 1 µM dex for 3 h before the addition of RSV. The effects of dex on IL-8 and MIP-1α release are shown in the left and right panels, respectively. For IL-8, n = 4, and for MIP-1α, n = 5. * p < 0.05.](http://www.jimmunol.org/)
85% inhibition of IL-8 even when dex was added 4 h after RSV stimulation.

**RSV stimulates induction of chemokine mRNA**

To determine whether RSV-induced chemokine production is transcription dependent and to determine whether the inhibitory effects of dex are at the level of mRNA synthesis, we isolated RNA from neutrophils and performed RPA to evaluate chemokine mRNA at 1 and 3 h after RSV stimulation. Figure 6A shows that, at 1 and 3 h after stimulation with RSV, mRNA levels for all three chemokines were increased above unstimulated neutrophils, and actinomycin-D (10 μg/ml) inhibited the increase in mRNA at both time points whereas dex had no effect on RSV-stimulated mRNA synthesis. Fig. 6B shows that, when corrected for GAPDH mRNA, RSV induced higher increases in mRNA for MIP-1α and MIP-1β than for IL-8.

**Discussion**

In infants with RSV bronchiolitis, neutrophils account for 93% and 76% of the inflammatory cells in the upper and lower airways, respectively (5). During RSV infection, the initial mediators (IL-6, IL-8, and RANTES) present in the airways are probably from epithelial cells (3). IL-8 will lead to the influx of neutrophils (20), which in turn can further contribute to the inflammation by the release of their own chemokines and granular enzymes. Our present study shows that RSV stimulates the release of the CC-chemokines MIP-1α and MIP-1β from neutrophils. MIP-1α stimulates eosinophil and basophil chemotaxis and degranulation (20, 21), which leads to the recruitment of these cells and the subsequent release of eosinophil cationic protein (ECP) and histamine in the airways. ECP and histamine have been shown in the respiratory secretions of infants with RSV bronchiolitis (22, 23).

In addition to ECP and histamine, IgE levels are also elevated in the nasal secretions of infants with RSV infection (23). MIP-1α enhances IgE production from human surface IgE-positive B cells (24). Our findings suggest that IgE production during RSV infection may be mediated by neutrophil-derived MIP-1α.

MIP-1β is another member of the CC chemokine family. Its function has not been extensively studied. MIP-1β does not induce chemotaxis of basophils or eosinophils, nor does it stimulate basophil histamine or leukotriene C4 release (20, 21). In a study that evaluated the effects of CC-chemokines on lymphocyte proliferation, Taub reported that MIP-1β stimulates Ag-specific Th2 lymphocyte proliferation and also up-regulates B7-1 on APCs (25). Thus, RSV-stimulated release of MIP-1β from neutrophils may enhance the proliferation of existing Th2 lymphocytes in the airways.

Neutrophils, IL-8, and MPO have also been found in respiratory secretions of children with virally induced asthma (9). This suggests a role for neutrophil degranulation in the pathophysiology of viral respiratory infections. We demonstrated that RSV stimulated MPO release, which is a marker for the degranulation of neutrophil primary granules. The primary granules of neutrophils also contain various enzymes, including elastase, collagenase, cationic proteins, and lysozyme, that are known to damage lung tissue (26). Of particular relevance to RSV-induced airway inflammation is elastase. This neutrophil granular enzyme induces mucus secretion from goblet cells (27, 28), a major finding in asthma and RSV bronchiolitis (29). Although we did not specifically measure elastase in our study, our finding that RSV stimulated MPO release would suggest that RSV would also stimulate elastase release. The RSV-induced MPO release, as well as chemokine release, from neutrophils was noncytotoxic, as evidenced by trypan blue exclusion.

Konig et al. showed that RSV-induced IL-8 production from neutrophils was transcriptional in nature (17). We found that the mechanism of increase in MIP-1α and MIP-1β was also at the
transcriptional level. RSV increased mRNA for these two cytokines at both 1 h and 3 h after stimulation. Preincubation with actinomycin-D, an RNA polymerase inhibitor, reduced RSV-stimulated MIP-1 and MIP-1α mRNA to the level of unstimulated neutrophils. This suggests that RSV stimulates the production of new mRNA rather than stabilizes the baseline mRNA in unstimulated neutrophils.

Konig showed that viable RSV is not necessary for stimulation of neutrophil IL-8 release (17). However, our data did not confirm these previous results. We found that viable RSV was necessary for the stimulation of neutrophil chemokine release. The persistence of stimulatory activity of RSV stock after inactivation with UV radiation or with heat in the studies of Konig et al. may be due to LPS contamination, for which the authors did not assess (17). In contrast to chemokine release, it appears that viable RSV is not absolutely necessary for stimulation of neutrophil degranulation. UV inactivation did not significantly reduce MPO release, and heat inactivation reduced release by only 32%. These data suggest that, while RSV infection of the neutrophils is required for chemokine release, interaction of RSV proteins or aggregates with the neutrophil cell membrane may be adequate for the induction of degranulation. The difference in potency of UV-RSV and H-RSV in the stimulation of degranulation might be explained by the difference in how the two treatments affect the virus. Whereas UV treatment affects only the infectivity of the virus, heat inactivation also changes the conformational structures of the viral proteins.

Dex has been shown to inhibit IL-8 release from LPS-stimulated neutrophils (15). Our study demonstrated that dex was able to significantly inhibit IL-8 release by 87% and MIP-1α release by 45% in RSV-stimulated neutrophils. Since the amount of chemokine mRNA synthesized was not remarkably different in the presence or absence of dex, whether the inhibition of chemokine protein release from RSV-stimulated neutrophils is at a posttranscriptional level deserves further exploration.

In contrast, dex did not inhibit neutrophil degranulation of MPO. Previous clinical studies have shown that corticosteroid therapy is not effective in treating patients with RSV bronchiolitis (12). Our finding that dex cannot inhibit all the mediators released from neutrophils may partially explain the inconsistency in clinical response to corticosteroids in patients with RSV bronchiolitis. Furthermore, in natural infection, corticosteroids are usually not administered until late in the course of the disease when inflammation has already been established.

Airway inflammation in RSV bronchiolitis is undoubtedly a multicellular process with epithelial cells, cytotoxic T cells, eosinophils, and neutrophils involved. Further studies are required to completely understand the mechanism of cell entry and mechanism of inflammation in RSV bronchiolitis and thus acquire insights into the effective treatment of RSV bronchiolitis.

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