

# Macrophage inflammatory protein-1 $\alpha$ and RANTES are present in nasal secretions during ongoing upper respiratory tract infection

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Macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) and RANTES (regulated upon activation, normal T-cell expressed and secreted) were measured by enzyme-linked immunosorbent assay (ELISA) from virus-infected respiratory cell culture supernatants and from 100 nasal wash samples obtained from patients aged 8 d to 10 yr. The results of the nasal wash samples were analyzed in relation to the etiology of the viral infection. *In vitro*, respiratory syncytial virus (RSV) induced the production of MIP-1 $\alpha$ , while both RSV and adenovirus were associated with the production of RANTES. Both MIP-1 $\alpha$  and RANTES were detected in nasopharyngeal secretions from pediatric patients with acute upper respiratory tract RSV, adenovirus, influenza, and parainfluenza virus infection ( $p < 0.001$  by Fisher's exact test). As both of these chemokines have potent effects on the recruitment and degranulation of eosinophils and basophils, further understanding of their role in upper respiratory tract infections may provide valuable insights into the immunopathogenesis of respiratory viral infections.

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Chemotactic cytokines (chemokines) participate in innate and adaptive immune responses by mediating leukocyte recruitment and activation (1). A common cause of acute airway inflammation, particularly in children, is viral infection. The cellular sources and mechanisms of cytokine induction have not been completely defined, but viruses are likely to cause inflammation, in part, by direct induction of host cell chemokines. We have recently demonstrated that severe respiratory syncytial virus (RSV) bronchiolitis is associated with elevated lower airway concentrations of the chemokines macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) and RANTES (regulated upon activation, normal T-cell expressed and secreted), and biologically active eosinophil granule proteins (2). The presence of immunoreactive and biologically active eosinophil secretory proteins in these secretions provides evidence for the role of eosinophils in the pathogenesis of

RSV disease, and suggests a role for MIP-1 $\alpha$  and RANTES as eosinophil chemoattractants *in vivo*. The present study was undertaken to determine if MIP-1 $\alpha$  and RANTES are produced and secreted in response to infection with other common pediatric respiratory viruses, both *in vitro* and *in vivo*.

MIP-1 $\alpha$  and RANTES are small, pleiotropic chemoattractants of the CC-chemokine family, which have been shown to recruit and/or activate monocytes, eosinophils, basophils, and several lymphocyte subpopulations (3–7). In this work, we showed that respiratory epithelial cells (HEp-2) and embryonic lung fibroblasts (MRC-5 and WI-38) responded to RSV infection by producing MIP-1 $\alpha$ , and responded to adenovirus infection by producing MIP-1 $\alpha$  and RANTES. We also examined the nasal respiratory secretions of 100 pediatric patients, who underwent nasal washes for suspected viral infection, and found that

MIP-1 $\alpha$  and RANTES were also expressed and secreted *in vivo* during acute upper respiratory tract infection with RSV, influenza, parainfluenza, and adenovirus.

## Materials and methods

### Cell culture

HEp-2 (human laryngeal carcinoma), WI-38 (human embryonic lung fibroblasts), and MRC-5 (human embryonic lung fibroblasts) cell lines were obtained from the American Type Tissue Collection (ATCC, Rockville, MD, USA) and cultured in Eagle's Modified Essential Medium (EMEM; Life Technologies Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin (Life Technologies Gibco BRL), at 37°C in 5% CO<sub>2</sub>.

### Preparation of viral suspensions

RSV-B (ATCC VR-1401), herpes simplex virus-1 (HSV-1; ATCC VR-260), adenovirus 21 (ATCC VR-1099), and cytomegalovirus (CMV) AD169 (ATCC VR-538), were obtained from the ATCC. A clinical rhinovirus isolate was the generous gift of our Clinical Virology Laboratory, SUNY HSC (Syracuse). RSV-B and adenovirus type 21 were used to inoculate 180-cm<sup>2</sup> flasks containing semi-confluent monolayers of HEp-2 cells. CMV AD169 and HSV-1 were used to inoculate, separately, semi-confluent monolayers of MRC-5 cells in 50 ml of maintenance media. When the cytopathic effect reached  $\approx 80\%$  (at 72–96 h), the culture supernatants were harvested and cellular debris was removed by centrifugation at 500 g. Aliquots of the virus suspensions were flash frozen and stored at  $-80^{\circ}\text{C}$ . Infectivity, determined by quantitative shell vial assay (8, 9) ranged from  $10^5$  to  $1.5 \times 10^6$  infectious units/ml. Control suspensions were prepared from uninfected cell cultures in an otherwise identical fashion. The cytopathic effect (CPE) was confirmed for rhinovirus by acid sensitivity, and by immunofluorescence staining of shell vials for each of the other types of virus.

### Virus infection of HEp-2, MRC-5, and WI38 cells

When cell monolayers were  $\approx 80\%$  confluent ( $15 \times 10^6$  cells/30 ml, or  $5 \times 10^5$  cells/ml), the medium was replaced (30 ml) and the cells were inoculated with virus suspension at a multiplicity of infection (MOI) of  $\approx 1$ . A second flask of each cell line, at the cell density indicated, was exposed to conditioned media as a control. Aliquots

(0.5 ml) of culture supernatants were removed at the time of infection ( $t=0$ ), and every 24 h thereafter. Cellular debris was removed by centrifugation (500 g for 5 min) and aliquots were stored at  $-80^{\circ}\text{C}$ . The concentration of MIP-1 $\alpha$  and RANTES in each aliquot was determined by enzyme-linked immunoassay (EIA; R&D Systems, Minneapolis, MN, USA).

### Collection and preparation of upper respiratory tract specimens

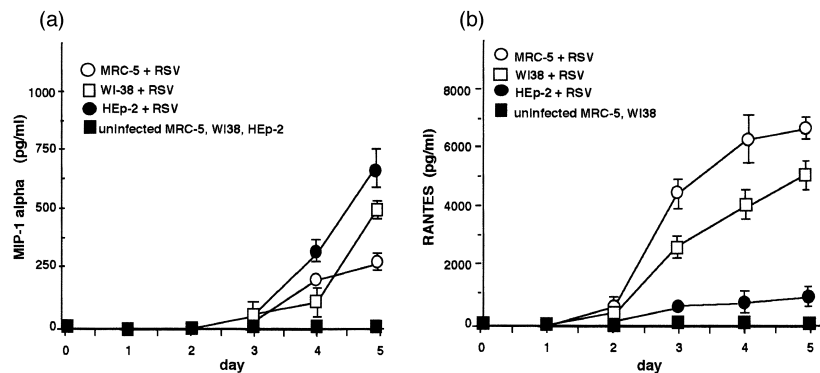
Nasal washes at our institution are routinely performed, as described below, for the diagnosis of suspected respiratory viral infection. The child is placed in the lateral decubitus position (so that one nostril is superior, and the other inferior), and 3–5 ml of sterile PBS is gently instilled into the superior nostril, in 1–2 ml aliquots, over  $\approx 20$  s. Secretions and lavage fluid are aspirated gently into a suction trap (routinely 1–2 ml in total). The suctioned upper airway secretions are placed on ice and transported to our Clinical Virology Laboratory where routine viral respiratory cultures are performed. In this study, the remainder of the sample was diluted with an equal volume of phosphate-buffered saline containing 4-(2-amino ethyl) benzene sulfonyl fluoride (AEBSF) and 2% mucocil. The specimen was clarified by centrifugation and stored in aliquots at  $-80^{\circ}\text{C}$ . Protein concentration was determined by the Bradford microassay (Bio-Rad, Richmond, CA, USA) against bovine serum albumin standards (Sigma Chemical Corporation, St. Louis, MO, USA). All nasal wash samples obtained between November 1997 and April 1998, which contained at least 0.5 mg/ml total protein, were analyzed. MIP-1 $\alpha$  and RANTES concentrations were determined in duplicate by enzyme-linked immunosorbent assay (ELISA; R&D Systems); results are expressed as pg/ml/mg total protein. This study was approved by our Institutional Review Board as an exempted protocol.

## Results

### MIP-1 $\alpha$ and RANTES production by RSV-infected epithelial and fibroblast cell lines

MIP-1 $\alpha$  and RANTES production, in response to ongoing RSV infection, was assessed by EIA of supernatant fractions harvested on d 1–5 after viral inoculation. Production of RANTES and MIP-1 $\alpha$  was observed in response to RSV infection in all three cell lines, with concentrations above background levels detected at d 3–5 and d 4–5, respectively ( $p < 0.01$ , Fig. 1a,b).

**Fig. 1.** Chemokine expression by MRC-5, WI-38, and HEp-2 cells in response to respiratory syncytial virus (RSV) infection. (a) Macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) (pg/ml) from RSV-infected cell culture supernatants. Concentrations detected differed significantly from uninfected control levels at d 3–5 for each cell type ( $p < 0.001$ ). (b) RANTES (pg/ml) from RSV-infected cell culture supernatants corrected for background RANTES production. \*Concentrations detected differed significantly from background levels at d 2–5 for each cell type ( $p < 0.001$ ).



MIP-1 $\alpha$  and RANTES production in response to ongoing infection with RSV, adenovirus, CMV, HSV-1, and rhinovirus *in vitro*

MIP-1 $\alpha$  and RANTES production in response to infection with each virus is shown in Fig. 2. The increase in MIP-1 $\alpha$  production was found to be unique to RSV infection in the cell lines tested (Fig. 2a). Increased expression of RANTES was observed in response to ongoing infection with adenovirus and RSV (Fig. 2b). No MIP-1 $\alpha$  and RANTES response was elicited by CMV, HSV-1, or rhinovirus, in any of the cell lines tested. As influenza and parainfluenza viruses do not reliably infect these cell lines, *in vitro* studies with these viruses were not performed.

#### Viral diagnostic testing

Indications for nasal washings and viral cultures were determined by the attending and resident physicians. Thorough viral diagnostic testing on nasal wash specimens was performed using traditional roll-tube cultures. In addition, analysis using commercially available solid-phase ELISAs was performed for RSV (Testpack; Abbott Diagnostics, Abbott Park, IL, USA) and/or influenza (Directogen; Becton-Dickinson Sparks, MD, USA), if requested by the attending physician. One-hundred nasal wash samples were evaluated. Of these, 39 were from patients with no virus isolated or detected by ELISA, and 61 were from patients with documented viral infection (40 RSV, 11 influenza, four parainfluenza, three adenovirus, two rhinovirus, and one HSV).

#### MIP-1 $\alpha$ and RANTES concentrations in nasal wash samples

MIP-1 $\alpha$  concentrations (pg/ml/mg protein) from 100 nasal wash samples are shown in Fig. 3. MIP-1 $\alpha$  was detected in all specimens from adenovirus-, influenza-, and parainfluenza-infected patients, and in 39/40 specimens from RSV-infected patients. RANTES was detected in 41/60 cul-

ture-positive washes. Seventeen of 40 culture-negative specimens had detectable RANTES levels, but only one was greater than 55 pg/ml/mg protein. Only three of 39 culture-negative samples had detectable MIP-1 $\alpha$ : two had very low levels of 4 and 5 pg/ml/mg protein; the third had 375 pg/ml/mg protein. Interestingly, the single specimen with a high MIP-1 $\alpha$  concentration also had a high RANTES concentration (366 pg/ml/mg protein; Fig. 4). Data was treated categorically (positive or negative viral diagnostics, detectable or non-detectable chemokine level), and demonstrated that the presence of MIP-1 $\alpha$  was significant at  $p < 0.001$ , and the presence of RANTES was significant at  $p < 0.05$ , for adenovirus-, influenza-, parainfluenza-, and RSV-infected samples.

#### Discussion

We show here that human respiratory epithelial cells and fibroblasts in culture respond to RSV infection by synthesizing and secreting both MIP-1 $\alpha$  and RANTES, and respond to adenovirus infection by synthesizing and secreting RANTES only. We showed, subsequently, that both MIP-1 $\alpha$  and RANTES were present in upper airway secretions of patients with upper respiratory tract infections caused by RSV, influenza, parainfluenza, and adenovirus. In contrast, infection with HSV or rhinovirus did not elicit this response, either *in vitro* or *in vivo*.

Although MIP-1 $\alpha$  was reported as absent in RSV-infected cell culture supernatants in an earlier report (10), we (2, 11) and others (12) have since reported the production of this chemokine *in vitro* in response to infection with RSV. Our *in vivo* results suggest that the involvement of MIP-1 $\alpha$  in the mucosal immune response to viral infection is not restricted to RSV, but rather also occurs during adenovirus, influenza, and parainfluenza virus infections. While it is clear that MIP-1 $\alpha$  and RANTES

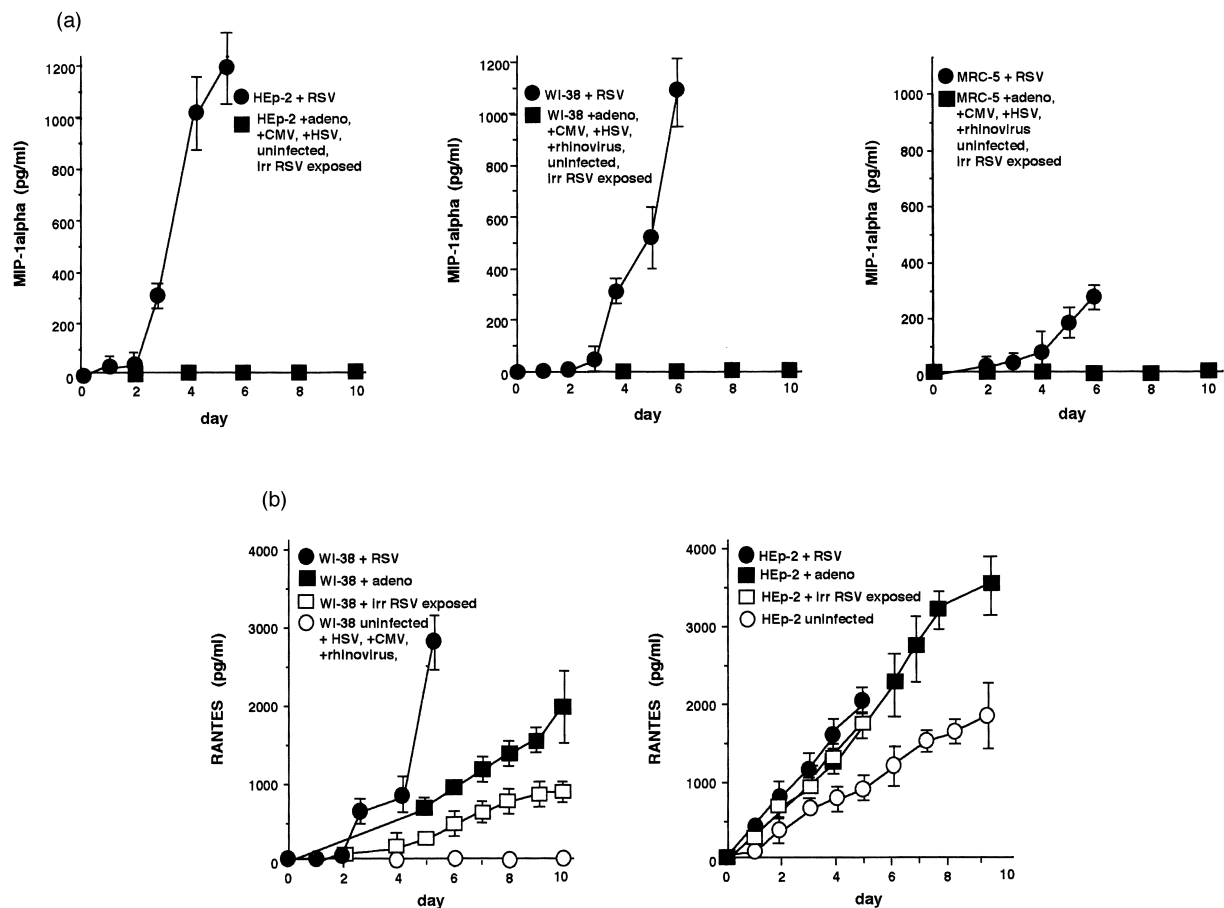


Fig. 2. (a) Macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) expression by HEp-2, WI-38, and MRC-5 cells in response to infection with respiratory syncytial virus (RSV), adenovirus, herpes simplex virus-1, cytomegalovirus, and rhinovirus. The concentration of MIP-1 $\alpha$  detected differed significantly on d 3 and thereafter for RSV-infected supernatants ( $p < 0.01$ ). (b) RANTES (pg/ml) from virus-infected WI-38 culture supernatants. The concentration of RANTES detected differed significantly from background levels on d 3 and thereafter for RSV-infected supernatants, and on d 5 and thereafter for adenovirus.

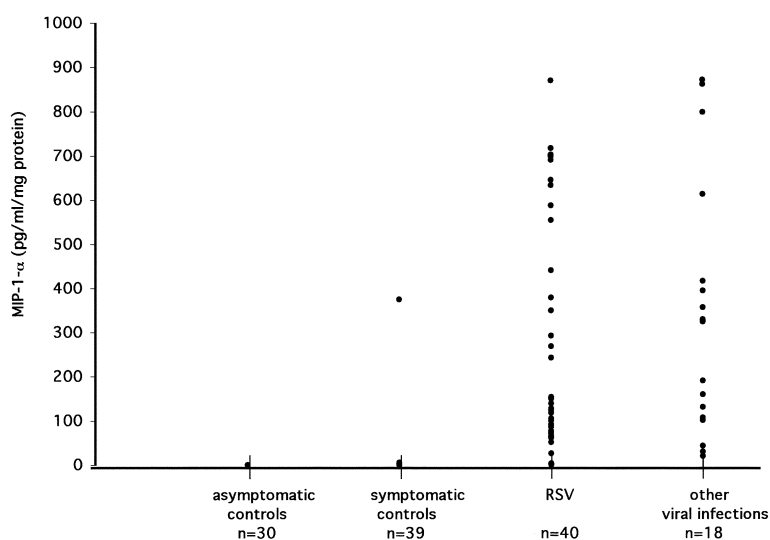


Fig. 3. Macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) concentrations (pg/ml/mg protein) from nasal wash samples obtained from patients with upper respiratory tract viral infections. Data was treated categorically ( $\pm$  specific virus, detectable/undetectable MIP-1 $\alpha$  levels). Fisher's exact test revealed a  $p < 0.001$  for adenovirus, influenza, parainfluenza, and RSV when compared with culture-negative controls.

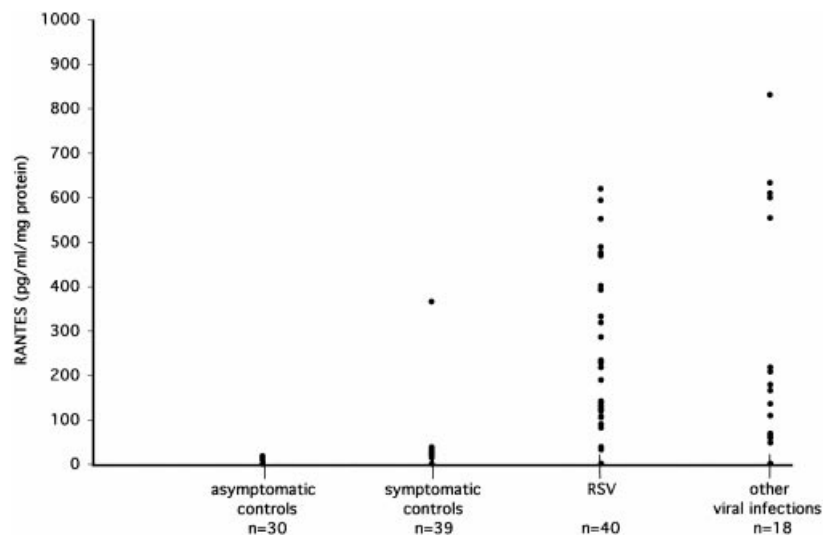


Fig. 4. RANTES concentrations (in pg/ml/mg protein) from nasal wash samples obtained from patients with upper respiratory tract viral infections. Data was treated categorically ( $\pm$  specific virus, detectable/undetectable RANTES levels). Fisher's exact test revealed a  $p < 0.001$  for adenovirus, influenza, parainfluenza, and RSV when compared with culture-negative controls.

expression can be induced by RSV in epithelial cells, it is unlikely that, *in vivo*, the epithelium is the only source of these chemokines. MIP-1 $\alpha$  production has been reported to be a response of isolated macrophages (13) and of epithelial cells (14) to infection with influenza A, and of both macrophages (15) and human T-cell lines (16) to infection with human immunodeficiency virus-1 (HIV-1).

Our results demonstrate that infection with common respiratory viruses is associated with production and secretion of MIP-1 $\alpha$  and RANTES. These chemokines are potent chemoattractants for human eosinophils (4), cells that have been associated with the inflammatory response to viral infections (17–19). Eosinophils are generally perceived as villains in viral-induced bronchospastic disease, as they release substances that induce bronchospasm (12, 18, 19). We have recently shown, however, that isolated human eosinophils mediate the direct destruction of extracellular virions of RSV and parainfluenza *in vitro* via the actions of their secretory ribonucleases, eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP), and that recombinant human EDN can act alone in promoting this anti-viral effect (8). Nasal ECP concentrations have been shown to be associated with severity of RSV disease (17, 19). Therefore, MIP-1 $\alpha$  and RANTES may be inducing chemoattraction and degranulation of eosinophils during infections with the other common pediatric respiratory viruses. Future studies in our laboratory are designed to determine if nasal ECP, or other eosinophil granule constituents, are also present during upper respiratory infections caused by influenza, parainfluenza, and adenovirus.

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