

Rhinovirus infection up-regulates eotaxin and eotaxin-2 expression in bronchial epithelial cells

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Summary

Background Human rhinoviruses (RVs) are the most common precipitants of asthma exacerbations. RV infection of bronchial epithelium results in local airway inflammation inducing eosinophil recruitment and activation. Induction of eosinophil chemoattractants could represent a central mechanism, as well as a prime target for intervention.

Objective To assess the effect of RV infection on mRNA expression and production of eosinophil chemoattractants by bronchial epithelial cells *in-vitro*.

Methods BEAS-2B cells were infected with major and minor RVs and the mRNA expression of IL-8, RANTES, MIP-1 α , eotaxin, eotaxin-2, MCP-2, MCP-3 and MCP-4 was assessed by reverse transcription PCR. In cases where mRNA induction was observed, a fluoroimmunoassay was used to confirm protein production. To assess the virus-specificity of the observed reactions, cells were also exposed to inactivated RVs.

Results RV infection was able to up-regulate mRNA expression of IL-8, RANTES, MIP-1 α , eotaxin and eotaxin-2, did not affect MCP-4, while MCP-2 and MCP-3 were not expressed either at baseline or after virus infection. Protein production was confirmed for IL-8, RANTES and eotaxin, but not for MIP-1 α . When RVs were inactivated cytokine up-regulation was almost completely lost.

Conclusion Infection of bronchial epithelial cells with RVs results in the production of a wide array of mediators that are able to chemoattract eosinophils. These include the eosinophil-specific molecules eotaxin and eotaxin-2, in addition to IL-8 and RANTES, which are the most abundant. Eosinophil recruitment after RV infection of bronchial epithelium could represent a central event in the pathogenesis of virus-induced asthma exacerbations.

Keywords: rhinovirus, eosinophils, asthma, chemokines, interleukin-8, RANTES

Clinical and Experimental Allergy, Vol. 31, pp. 1060–1066. Submitted 17 July 2000; revised 5 December 2000; accepted 21 December 2000.

Introduction

It is now well established that upper respiratory tract viral infections are a major trigger of acute asthma exacerbations [1]. Among the viruses involved, human rhinoviruses (RVs) are the most frequently detected agents. However, the pathogenesis of virus-induced

asthma exacerbations is still incompletely understood. Recent evidence indicates that RVs are able to infect the lower airway epithelium and generate a local inflammatory response [2]. Since the airway epithelium is the location of the initial host–virus interaction, it is likely that local release of inflammatory mediators could be a first step in the inflammatory cascade leading to an asthma exacerbation.

Among cells that are considered important in allergic inflammation, eosinophils have a predominant position.

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Table 1 Primer pairs, amplicon sizes and thermal cycling conditions for chemokine RT-PCRs

	Primer pair	Product size (bp)	Thermal cycles (denaturation, annealing, extension × cycle number)
IL8	ATGACTTCCAAGCTGGCCGTGGCT TCTCAGCCCTCTTCAAAAACCTCTC	289	94°-15'', 50°-30'', 72°-30'' × 28
RANTES	ATGAAGGTCTCCGCGGCACGCCTCGCTGTC CTAGCTCATCTCCAAAGAGTTGAT	252	94°-15'', 53°-20'', 72°-30'' × 38
MIP-1α	CAGGTCTCCACTGCTGCC CACTCAGCTCCAGGTCAC	252	94°-15'', 50°-20'', 72°-30'' × 40
Eotaxin	CCCAACCACCTGCTGCTTTAACCTG TGGCTTTGGAGTTGGAGATTTTGG	208	94°-30'', 60°-30'', 72°-30'' × 38
Eotaxin-2	GGAGTGGGTCCAGAGGTACA TTAGCAGGTGGTTTGGTTGC	124	95°-30'', 53°-30'', 72°-20'' × 34
MCP-2	ATGCTGAAGCTCACACCCTTGCCC CAGATGCTTCATGGAATCCCTGACC	305	95°-40'', 64°-40'', 72°-40'' × 45
MCP-3	ATTGCTGCGTGCTTGATG CTGCTTAGGGATTTTCTTATTGAT	307	95°-30'', 55°-30'', 72°-30'' × 40
MCP-4	CCCGCATGCAGCCAGATGCACTCAACG AAAGGATCCAGTCTTCAGGGTGTGAGC	215	95°-30'', 55°-60'', 72°-120'' × 31
β-actin	TGACGGGGTCACCCACACTGTGCCCATCTA CTAGAAGCATTGCGGTGGACGATGGAGGG	661	94°-20'', 50°-20'', 72°-30'' × 32

Evidence for a role of eosinophils in asthma comes from a variety of sources. These cells are present in increased numbers in bronchial biopsies, bronchoalveolar lavage (BAL) fluid and peripheral blood of patients with mild to moderate asthma, and the degree of eosinophilia correlates with the severity of the disease, as well as with bronchial hyper-responsiveness. Furthermore, eosinophil granule proteins, such as major basic protein (MBP), eosinophil cationic protein (ECP) and more, have been extensively correlated with symptoms of asthma [3].

It is therefore of interest to assess whether virus-induced epithelial activation may be involved in the recruitment and/or activation of eosinophils [4], a mechanism that could represent an important step in the pathogenesis of virus-induced asthma. The prime candidates mediating such a process could be expected to be among the growing family of chemokines, several of which have been shown to be important in eosinophil trafficking. Certainly, interleukin (IL)-8 and more recently RANTES, both of which can act on eosinophils, are induced by RV infection of respiratory epithelium [5,6]. Nevertheless, information on RV-mediated epithelial secretion of the increasing number of eosinophil chemoattractants and activators is limited. Therefore, in this study we used BEAS-2B cells, a well-established *in-vitro* lower respiratory epithelium model of RV infection [7], to examine the mRNA expression and protein production of a wide array of mediators that are involved in eosinophil

recruitment and activation, after infection with major and minor type RVs.

Materials and methods

Viruses

RV1b, a minor RV subtype was originally obtained from the Medical Research Council (MRC) Common Cold Unit (Salisbury, UK). RV16, a major RV subtype, was a generous gift of Drs William Busse and Elliott Dick (Madison, WI, USA). The identity of the viruses was confirmed by neutralization with specific antisera (American Type Culture Collection, ATCC, Rockville, MD, USA). Viruses were propagated in large quantities in Ohio HeLa cells, at 33 °C, in a humidified, 5% CO₂ incubator. When full cytopathic effect (CPE) developed, cells and supernatants were harvested, pooled, frozen and thawed twice, clarified, sterile-filtered, aliquoted and stored at -70 °C. Virus titrations were performed on Ohio HeLa cell monolayers as previously described [8]. For each experiment, a new vial was rapidly thawed and used immediately.

Cell cultures

BEAS-2B, a continuous bronchial epithelial cell line was obtained from the ATCC. Cells were cultured in Eagle's

MEM with Glutamax (Gibco BRL, Paisley, UK) supplemented with 2% Ultrosor-G (Gibco) and 40 µg/mL of gentamycin, in a humidified 5% CO₂ incubator at 37 °C. Cells were split twice weekly.

In vitro infection of respiratory epithelial cells

BEAS-2B cells were plated in 12-well plates (Falcon Primaria, Becton Dickinson, Mountain View, CA, USA) until reaching 80% confluence. They were then exposed to 1 MOI (multiplicity of infection – infectious units/cell) of virus, for 1 h at room temperature with gentle shaking, after which the virus was washed and the medium replaced. This dose was found to be optimal for chemokine studies in initial dose–response experiments. Non-infected Ohio HeLa cell preparations processed in parallel with virus-infected preparations were used as negative controls. Eagle's MEM supplemented with 1% Ultrosor-G, 1% MgCl and 4% tryptose phosphate broth (Sigma Chemical Co., Poole, UK), without antibiotics was used for the experiments. Incubation took place in a humidified 5% CO₂ incubator at 33 °C. Supernatants and cell lysates were harvested at various time-points and stored at –80 °C until assayed.

Effect of RV-inactivating factors

In order to assess the specificity of RV-mediated responses, RV preparations were exposed to 58 °C for 1 h, to 1200 mJ/cm² of UV light, or to 1 mg/mL of soluble intercellular adhesion molecule-1 (sICAM-1, kindly donated by P. Esmon, Bayer, Berkley, CA, USA) for 45 min at room temperature. These suspensions containing inactivated RV, as assessed by lack of RV replication in Ohio-HeLa cells, were used to infect BEAS-2B monolayers in parallel with non-inactivated positive controls.

Mediator mRNA expression

RNA was extracted from BEAS-2B cell monolayers as follows. Two hundred µL of Trizol™ reagent (Gibco) were added directly on cultured monolayers after removal of the supernatant and the cell lysate was transferred in eppendorf tubes. After 5 min at room temperature, 40 µL of chloroform were added; tubes were shaken vigorously for 15 s and further incubated for 2–3 min at room temperature. They were then centrifuged at 12 000 g for 15 min, the aqueous phase was removed and the RNA was precipitated with isopropanol, washed with 75% ethanol, air dried and resuspended in 10 µL of ultra-high quality water (UHQ). Reverse transcription was performed in a 50-mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂ buffer, with 10 mM DTT, 0.4 mM dNTPs, 0.5 µg random hexamer primers (Promega Corporation, Southampton, UK) and 100 U of reverse

transcriptase (Superscript™, Gibco). The mix was incubated at 37 °C for 60 min to yield cDNA.

Semi-quantitative RT-PCRs were performed on cDNA from BEAS-2B lysates, in a total volume of 50 µL, with 1.5 mM Mg²⁺, 0.2 mM dNTPs, 2.5 U Taq DNA polymerase (Qiagen, Crawley, UK) and 0.5 µM of primers. IL-8, RANTES, MIP-1α, MCP-2, MCP-3, MCP-4, eotaxin, and eotaxin-2 were examined. Primer pairs and thermal cycling conditions are shown in Table 1. Parallel PCR amplification of β-actin mRNA was used as a control of cDNA loading. Densities of PCR bands were measured by Band Leader™ V3.00 software and expressed as a percentage of their respective β-actin value.

Protein production

Levels of eosinophil chemoattractants were measured in culture supernatants using a fluoroimmunoassay. Low fluorescence immunoassay plates (Fluoronunc, Nunc) were coated overnight at room temperature with the appropriate antibody solution (R & D Systems, Abingdon, UK) at a concentration of 4 µg/mL. After blocking non-specific binding sites with 1% bovine serum albumin (BSA), 5% sucrose in PBS (Sigma), standards and appropriately diluted samples were added and incubated at 4 °C overnight, followed by incubation with a biotinylated secondary antibody for 1 h at 37 °C. Biotinylated antibody concentrations were 20 ng/mL for IL-8, 5 ng/mL for RANTES and 40 ng/mL for MIP-1α and 90 ng/mL for eotaxin (R & D). Positive samples were detected by incubation with a europium-streptavidin conjugate (DEL-FIA®, EG & G, Milton Keynes, UK) for 1 h at 37 °C, followed by DELFIA® Enhancement solution (EG & G) for 15 min at room temperature with gentle shaking. This reagent dissociates europium from the solid phase into solution, to form highly fluorescent chelates with ligands present in the solution. Europium-mediated fluorescence was measured with a DELFIA 1234 fluorometer (Wallac). The assay volume was 80 µL in all cases. Wells were washed four times between each of the described steps. The sensitivity of the assays was 4 pg/mL for IL-8 and RANTES, 10 pg/mL for MIP-1α and 16 pg/mL for eotaxin.

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Comparisons of means were done by the Wilcoxon non-parametric matched pair test. Calculations were performed with the Statistica 4.5 for Windows software package. Values of *P* < 0.05 were considered significant.

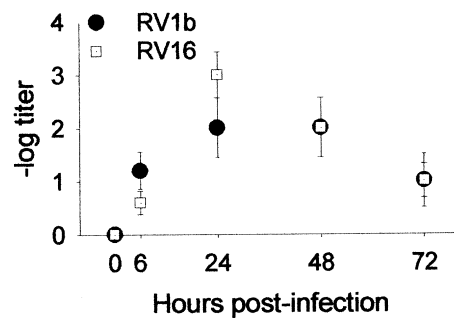


Fig. 1. Infection of BEAS-2B cells with RV1b (closed circles) and RV16 (open squares). Viral titres, which are not detectable just after the exposure, become detectable 6 h later, further increase for 24–48 h, and slowly decline thereafter. Results are mean $\pm$ SEM of three–five experiments.

Results

Infection of BEAS-2B cells with RVs

Productive infection of BEAS-2B cells with RVs was confirmed by sequential titrations of cell lysates 1, 6, 24, 48 and 72 h after exposure to the viruses ($n = 3–5$). No virus could be detected 1 h after exposure. Virus titres were measurable 6 h later, peaking at 24 h after exposure, and slowly declining thereafter. Both RV strains showed the same pattern of infection (Fig. 1), while no RV-mediated cytopathic effect was observed in these cultures.

Effects of RV infection on chemokine mRNA expression

Exposure of BEAS-2B cells to RVs was able to significantly up-regulate mRNA expression of IL-8, RANTES, MIP-1 α and eotaxin-2 (Fig. 2). Maximum mRNA expression was observed 48 h post-infection for all these mediators and this effect was similar for both minor (RV1b) and major (RV16) rhinoviruses. Eotaxin mRNA was marginally up-regulated at 24 h post-infection (control 21% $\pm$ 6.4, RV1b 32% $\pm$ 7.3, RV16 39% $\pm$ 9.1, $n = 7$, $0.05 < P < 0.1$). In contrast, mRNA expression of MCP-2 and MCP-3 was not detected in the cultures, either before or after RV infection, while MCP-4 mRNA was present but was not affected by RV infection (data not shown).

Effects of RV infection on chemokine protein production

Levels of IL-8 and RANTES were elevated 24 h and 48 h after exposure to RVs, increasing with time (Fig. 3). Increased levels of IL-8 and RANTES continued to be present in the supernatants at 72 h and 96 h post-infection (data not shown). Eotaxin was significantly higher in the supernatants from the infected cultures 24 h post-infection (Fig. 3). RV1b was more potent than RV16 in inducing chemokine production. In contrast, MIP-1a was not detectable in the supernatants.

Inactivation experiments

Exposure of RVs to high temperature or UV light was able

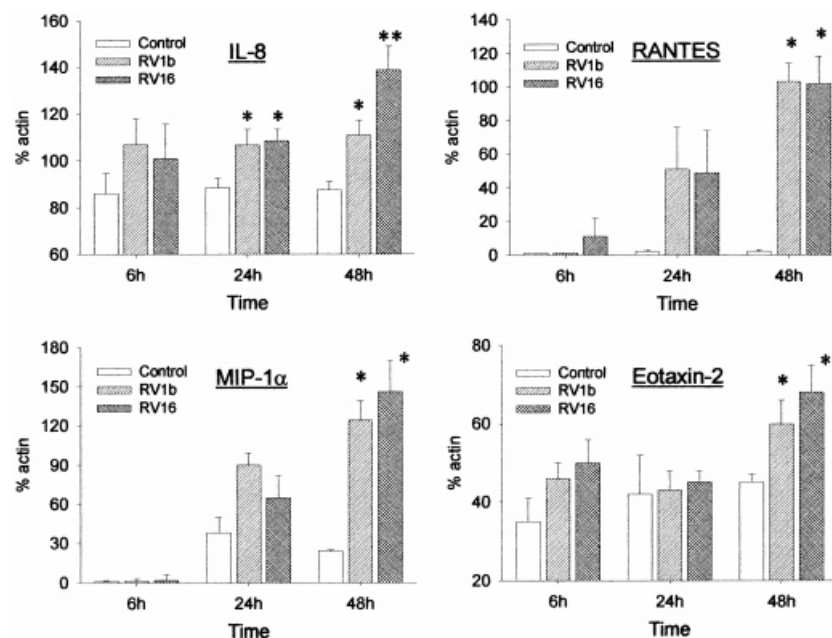


Fig. 2. Time-course of IL-8, RANTES, MIP-1a and eotaxin-2 mRNA expression, as a percentage of the relative beta-actin value, in bronchial epithelial cells infected with RV1b or RV16. Significant mRNA induction is observed for all the above mediators at 48 h post-infection. Results are mean $\pm$ SEM of four–ten experiments. * $P < 0.05$, ** $P < 0.01$.

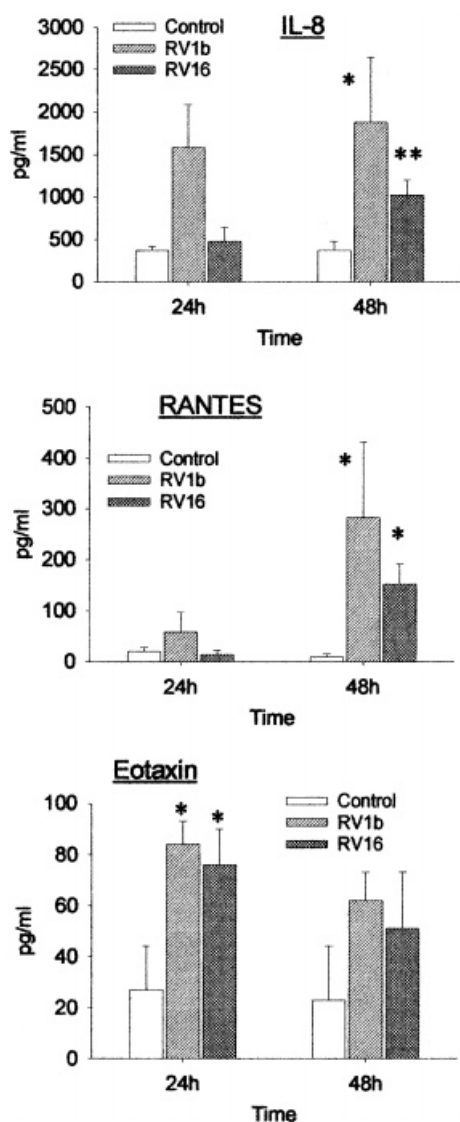


Fig. 3. Production of IL-8, RANTES and eotaxin 24 h and 48 h after exposure of BEAS-2B cells to RV1b and RV16. IL-8 and RANTES tend to increase with time, becoming statistically significant at the 48 h time-point, while eotaxin production peaks at 24 h. Results are mean $\pm$ SEM of four–seven experiments. * $P < 0.05$, ** $P < 0.01$.

to almost completely abrogate IL-8 and RANTES production in the cultures, confirming that the observed responses were specific to the viruses and not HeLa-derived factors. Exposure to ICAM-1, the receptor of major RV subtypes, inhibited only RV16, but not RV1b-mediated IL-8 and RANTES production (Fig. 4).

Discussion

Eosinophilic inflammation is a hallmark of asthma. Therefore, recruitment and activation of eosinophils by

RV-infected bronchial epithelium could represent an important mechanism involved in virus-induced asthma exacerbations, as well as a potential target for therapeutic intervention. Eosinophil infiltration has been documented in bronchial tissue after experimental upper airway RV infection [4]. An increasing number of mediators has been identified which have the ability to cause eosinophil migration and/or activation, including IL-8, RANTES, MCP-2, MCP-3, MCP-4, MIP-1a, eotaxin and eotaxin-2 [9,10]. We hypothesized that epithelial production of these mediators in response to virus infection was likely to be an important mechanism in the chemoattraction of eosinophils to the bronchial mucosa during virus-induced asthma exacerbations. To investigate this, the mRNA expression and protein production of these eosinophil chemoattractants and/or activators after in-vitro exposure of BEAS-2B cells to major and minor RVs were examined in this study. BEAS-2B cells are a well-characterized model for RV infection of bronchial epithelium; the characteristics of the infection and inflammatory response are strikingly similar between these cells and primary human bronchial epithelial cells [2,7]. Furthermore, RV infection of BEAS-2B cells results in the production of IL-6, GM-CSF and TNF- α that are also involved in eosinophil pathophysiology [11].

According to our results, RV infection was also able to up-regulate IL-8, RANTES, MIP-1a and eotaxin-2 mRNA; up-regulation of eotaxin mRNA was also present, although it did not reach statistical significance. The MCPs were not inducible by RV in these cells. Protein production of these mediators was confirmed for IL-8, RANTES and eotaxin, while MIP-1a levels were undetectable. Antibodies against eotaxin-2 were not available. Inactivation experiments confirmed that virus culture by-products did not interfere with chemokine production in this model. A notable finding of this study was the complete inhibition of IL-8 and RANTES production obtained with UV-light treatments of RV, which contrasts with previous evidence showing only partial inactivation and thus suggesting a receptor binding-mediated element in such production [5,6]. This discrepancy might be attributed to incomplete inactivation of RVs in previous studies or excessive UV treatment in this one; however, further detailed studies looking into the participation of receptor binding in RV-mediated inflammation are certainly required.

Production of IL-8 and RANTES from BEAS-2B cells after RV infection is in agreement with previous reports [5–7]. We have also recently shown that these mediators are produced after RV infection of primary bronchial epithelial cells [2]. Furthermore, IL-8, RANTES and MIP-1a have been found to be increased in nasal secretions of patients with virally induced asthma exacerbations [12]. MIP-1a protein was not detectable in the supernatants of our cultures, even though mRNA expression was present

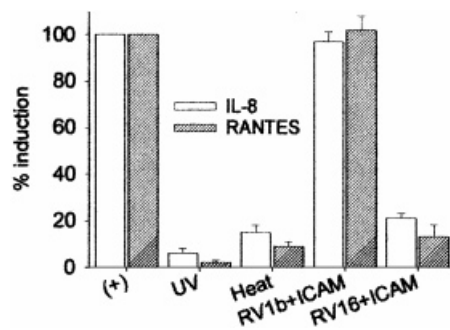


Fig. 4. Inhibition of rhinovirus-mediated IL-8 and RANTES production. Columns express cytokine up-regulation, relative to non-inactivated RV positive control (+) = 100%. Exposure to UV light (UV), and heat inactivation (Heat) almost completely abrogated RV-mediated IL-8 and RANTES up-regulation. RV1b was not affected by the addition of sICAM-1, in contrast to RV16 that uses this molecule as its cellular receptor and therefore was inactivated. Results are mean $\pm$ SEM of four experiments.

and up-regulated after RV infection. This chemokine may not be secreted by BEAS-2B cells or its levels may have been lower than the sensitivity of the assay. Nevertheless, MIP-1a is up-regulated in primary bronchial epithelial cells infected with respiratory syncytial virus [13].

The most important finding of this study is the induction of eotaxin and eotaxin-2 by RV infection of epithelial cells, an observation that has not been previously described. These eosinophil-specific chemoattractants are particularly interesting as they might be able to explain the selective recruitment of eosinophils in allergic inflammation. Eotaxin is produced by the airway mucosa, and local levels are elevated in allergic airway disease and reduced by steroid treatment [14]. Its chemotactic capacity for eosinophils has been confirmed after *in-vivo* administration [15] and elevated levels of eotaxin have been found in nasal secretions of RV-experimentally infected atopic volunteers [16]. Lately, it has been suggested that eotaxin, in contrast to eotaxin-2, might be involved in an early rather than the late accumulation of eosinophils in the skin of human atopic subjects [17]. This is compatible with our data, showing an early up-regulation of eotaxin, a time-course that differed from the other chemokines. Eotaxin-2 is also an eosinophil-specific C-C chemokine, acting through CCR-3. Its action on eosinophil and basophil leucocytes is similar to eotaxin and it is produced in the airway mucosa of asthmatic subjects [10]. Although the data on eotaxin-2 biological activities are still limited, in a study of nasal polyps local mRNA expression of this chemokine was higher than eotaxin or MCP-4 [14]. Furthermore, eotaxin-2 was shown to be the most potent between eosinophil chemoattractants [18]. It is therefore possible that eotaxin-2 could be important both as an

eosinophil-specific mediator and as a target for therapeutic intervention in virus-mediated as well as in allergic inflammation.

The relative potency of different chemokines in eosinophil recruitment has been the subject of several studies, with no conclusive results [9]. Certainly, the *in vivo* environment is much more complicated than *in vitro* models and the action of any specific set of chemokines would depend upon a multitude of factors, including timing of secretion, half-life, receptor affinity, presence of inhibitors, and more. Although IL-8 was the most abundant mediator in our cultures, there is an open debate on whether this chemokine can actually act on eosinophils [19,20]. In contrast, RANTES, which was also produced in significant concentrations in this and other studies, is a potent eosinophil chemoattractant, acting through CCR-3 and possibly through CCR-1. In comparison with RANTES, MIP-1a or MCP-3 are considered less potent, while eotaxin, eotaxin-2 and MCP-4 are of equal or higher potency. In considering this information together with our findings, it is possible that RANTES and the eotaxins might be the most relevant among the currently known mediators, in RV-induced eosinophil recruitment. It is of interest that IL-8, RANTES and eotaxin up-regulation in bronchial epithelium is mediated through the nuclear factor κ B (NF- κ B) [21–23]. Although there are currently no data available on the transcriptional pathways that mediate eotaxin-2 production, is it possible that NF- κ B could also be involved. This is supported by recent evidence showing that mice deficient in the p50 subunit of NF- κ B are incapable of mounting eosinophilic airway inflammation compared with wild-type mice [23]. Therefore, NF- κ B may be an important target for therapeutic intervention in eosinophilic inflammation.

In conclusion, when infected with human rhinoviruses, respiratory epithelial cells are able to express mRNA and produce several chemokines that can mediate eosinophil recruitment, including the eosinophil-specific chemokines eotaxin and eotaxin-2. As RVs are able to infect the lower airway epithelium [2], eosinophil chemotaxis through the mediators described herein may be a subsequent step in the pathogenesis of acute exacerbations of asthma.

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