

Rhinovirus Induction of the CXC Chemokine Epithelial-Neutrophil Activating Peptide-78 in Bronchial Epithelium

Howard Donninger,^{1,2,a} Richard Glashoff,^{1,2} Hans-Michael Haitchi,^{1,a} James A. Syce,¹ Reena Ghildyal,³ Estrelita van Rensburg,² and Philip G. Bardin³

¹Lung and Allergy Unit and ²Medical Virology, University of Stellenbosch, Cape Town, South Africa; ³Department of Respiratory and Sleep Medicine, Monash Medical Centre and University, Melbourne, Australia

Epithelial-neutrophil activating peptide-78 (ENA-78) induces neutrophil migration, an early response to viral infection. Rhinovirus serotype 16 (RV16) was used to infect primary bronchial epithelial cells and a cell line (BEAS-2B). Release of ENA-78 protein was measured by enzyme-linked immunosorbent assay, ENA-78 mRNA production was quantified by reverse-transcription polymerase chain reaction, and ENA-78 promoter activity was assessed by use of a promoter construct. After infection with RV16, ENA-78 protein and mRNA increased significantly, and RV16 induced 3-fold increases in ENA-78 gene transcription. Nasal ENA-78 measured in patients with asthma with and without RV infection was more elevated in patients with RV infection present. Our study demonstrates that ENA-78 is produced in bronchial epithelial cells in response to RV16 infection. With other chemokines, it may be an important initiator of neutrophil airway inflammation during RV common colds and thus may play a role in the development of virus-associated airway pathologies.

Common colds caused by respiratory viruses are a troublesome problem of everyday living, with the average person experiencing 1 or 2 infections per year. Recently, it has been recognized that common cold viruses are responsible for up to 80% of acute asthma attacks and

that they are also likely to cause the majority of exacerbations of chronic obstructive pulmonary disease (COPD) [1–3]. Therefore, prevention of virus-associated asthma and COPD exacerbations and the development of new antiviral compounds have become priorities.

The inflammatory pathways induced by common cold viruses are poorly understood. It is known that neutrophils are important inflammatory cells detected in the early stages of nasal infection with rhinovirus (RV) [4, 5], which is the cause of the majority of common colds. Peripheral neutrophilia also is often detected [6], and bronchial neutrophilia has been demonstrated in association with experimental RV infection [7]. Recruitment of neutrophils involves a series of events, including adhesion, transendothelial migration, and chemotactic movement, the latter principally mediated by chemokines. Recently, it has been demonstrated that chemokines, such as interleukin (IL)–8, are produced in response to RV infection of epithelium [8, 9], which suggests an important role for neutrophil chemoattractants in early events after RV infection.

Received 11 November 2002; accepted 6 January 2003; electronically published 15 May 2003.

Presented in part: Thoracic Society of Australia and New Zealand Annual Scientific Meeting, Cairns, Australia, 22–24 March 2002 (abstract A18).

Studies were approved by the Ethics Committee, University of Stellenbosch, Cape Town, South Africa, and Southern Health Human Research Ethics Committee, Monash Medical Centre, Melbourne, Australia.

Financial support: Allergy Society of South Africa, South Africa Medical Research Council, University of Stellenbosch, Cape Town, Monash University, and Asthma Victoria, Melbourne, Australia.

^a Present affiliations: National Cancer Institute, National Institutes of Health, Rockville, Maryland (H.D.); Respiratory Cell and Molecular Biology, Southampton General Hospital, Southampton, United Kingdom (H.-M.H.).

Reprints or correspondence: Dr. Phil Bardin, Dept. of Respiratory and Sleep Medicine, Monash Medical Centre, Clayton Road, Clayton, Victoria 3168, Australia (p.bardin@southernhealth.org.au).

The Journal of Infectious Diseases 2003;187:1809–17

© 2003 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2003/18711-0017\$15.00

Human epithelial-neutrophil activating peptide-78 (ENA-78; also referred to as CXCL-5) was first identified from the conditioned medium of the stimulated human type II epithelial cell line A549 [10]. ENA-78 belongs to the α -chemokine subfamily and, like IL-8, can induce neutrophil chemotaxis, increases in intracellular free calcium, and elastase release [10]. Its stimulatory activities on neutrophils appear to be mediated by the IL-8 type B receptor [11]. Recent studies have supported a role for ENA-78 in allergic airway inflammation [12] and idiopathic pulmonary fibrosis [13]. However, production by primary airway cells of this CXC chemokine in response to RV infection has not been examined, and it is unknown whether it is increased during human RV common colds.

We postulated that ENA-78 might be produced in response to RV infection of airway epithelial cells. We investigated whether bronchial epithelial cells secreted ENA-78 in response to RV infection and measured levels of this chemokine in nasal fluid during RV asthma exacerbations.

MATERIALS AND METHODS

Cell culture. BEAS-2B cells were obtained from ATCC (Rockville, MD) and were cultured in Dulbecco's modified Eagle medium (DMEM) F12 supplemented with 2% Ultrosor G (Gibco) and 0.5 μ g/mL epidermal growth factor (Sigma). Ohio strain HeLa cells (gift of S. Johnston, Imperial College, London) were cultured in Eagle's MEM supplemented with 10% fetal bovine serum. Primary bronchial epithelial cells were obtained from brushings carried out during routine diagnostic bronchoscopy. The cells were collected in MEM containing 0.1% dispase and 50 μ g/mL DNase (Sigma). Cells were washed, resuspended, and seeded into culture dishes (Falcon Primaria) in bronchial epithelial medium (BEGM; Clonetics) containing 5 mg/mL insulin, 0.5 μ g/mL epidermal growth factor, 10 mg/mL transferrin, 13 mg/mL bovine pituitary extract, 0.5 mg/mL hydrocortisone, 0.5 mg/mL epinephrine, 0.1 μ g/mL retinoic acid, and 6.5 μ g/mL triiodothyronine. Cultures were monitored regularly, and when cells reached ~50% confluence, culture medium was changed to a 50:50 mix of BEGM and DMEM F12 supplemented with Ultrosor G and epidermal growth factor. Cells were used after 2–3 passages at or near 100% confluence, and epithelial phenotype was confirmed by staining for cytokeratin (85%–90% purity).

Virus stock preparation. RV16 (gift of E. Dick and W. Busse, Madison, WI) was cultured in HeLa cells (Ohio strain). Virus stocks were inoculated onto HeLa cells and cultured until cytopathic effect was observed. Supernatant was collected, and remaining cells were scraped from the flask. Cell-associated virus was released from the cells by repeated freezing and thawing. Virus stock then was clarified by centrifugation. RV16 was

titrated by diluting stock virus (10^{-1} to 10^{-8}) and then inoculating it onto near-confluent HeLa cells in 96-well microtiter plates. After development of cytopathic effect, TCID₅₀ was determined by the Spearman-Kärber method. Ultrapurification of RV16 was not done, because previous studies have all demonstrated that similar cellular responses are elicited regardless of whether standard or purified virus stock is used [8, 14, 15]. UV-inactivated RV16 was prepared by irradiation at 254 nm wavelength in polypropylene containers for 40 min with a mercury germicidal lamp. IL-8 and ENA-78 were not detectable in the virus pool used to infect cells.

Cell infection and supernatant preparation. All cells were infected with RV in the same manner. Virus stock was diluted in growth medium and was added to the cells. Virus infection was done in small volumes (for a 25-cm² flask, 1 mL of inoculum was added). Cultures then were incubated for 1–2 h at 37°C on a shaker. Cultures were removed from the shaker, and additional culture medium was added. Cells were maintained at 37°C in a CO₂ incubator. Supernatants were collected at the appropriate time and were frozen at –20°C. At the time of ELISA, supernatants were thawed, and cell debris was removed by centrifugation. Control experiments included mock-infected cells (HeLa cells without RV16 infection) and UV-inactivated RV16.

Quantification of ENA-78 and IL-8 protein. ENA-78 and IL-8 were quantified by sandwich ELISA with paired antibody reagents (R&D Systems). ELISA plates (NUNC Maxisorb) were coated with capture antibody (anti-human IL-8 and ENA-78) at 4 μ g/mL in PBS overnight at 4°C. Plates then were brought to room temperature and blocked with blocking buffer (1% bovine serum albumin [BSA] in PBS) for 2 h. After washing, standards and samples were added to plates in duplicate. Standards (recombinant human IL-8 and ENA-78) were doubly diluted from 1000 pg/mL to 7 pg/mL in blocking buffer (with 0.05% Tween 20). Plates were incubated overnight at 4°C. After washing, biotinylated detection antibodies (100 ng/mL) were added and incubated at room temperature for 2 h. After washing, tetramethylbenzidine hydrochloride substrate (Sigma) was added, and after color development, the reaction was stopped with 0.1 M H₂SO₄. Absorbance was read at 450 nm, and chemokine concentrations were calculated from the standard curve.

Detection of ENA-78 and IL-8 mRNA. RNA was isolated from the bronchial epithelial cell lines and primary bronchial epithelial cells as described elsewhere [16]. Two micrograms of total RNA was used to transcribe cDNA with use of Moloney murine leukemia virus reverse transcriptase (Promega; Whitehead Scientific). The RNA was incubated in the presence of 1 mM dNTPs, 25 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, and 5 U of RNasin ribonuclease inhibitor (Promega) at 37°C for 1 h in a final volume of 20 μ L. The enzyme was heat-inactivated at 95°C for 5 min. Two microliters

of the cDNA was used in a polymerase chain reaction (PCR) to amplify regions of the IL-8-, ENA-78-, and β -actin-coding regions. Amplification was done in a final volume of 50 μ L in 1 \times *Taq* DNA polymerase reaction buffer (Promega) in the presence of 0.2 mM dNTPs, 2 U of *Taq* DNA polymerase (Promega), and 30 pmol of the following primers: IL-8 sense (5'-TCTGCAG-CTCTGTGTGAAGG-3') and IL-8 antisense (5'-AACCCTCTGC-ACCCAGTTTTC CTTG-3') for IL-8; ENA-F (5'-TTACAGACC-ACGCAGG-3') and ENA-R (5'-GGCTTC TGGATCAAGACA-3') for ENA-78; and β -F (5'-GTGGGGCGCCCCAGGCACCA-3') and β -R (5'-CTCCTAATGTCACGCACGATTTTC-3') for β -actin. Conditions used were an initial denaturation step of 94°C for 1 min, followed by 35 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s), and extension (72°C for 1 min). Five microliters of the amplification reaction was resolved on a 1.5% agarose gel, and the bands were visualized under UV light after the gel was stained with ethidium bromide (Sigma). The intensity of the bands was determined by image analysis (GeneTools), and the intensity of each band for IL-8 and ENA-78 was expressed relative to the intensity of the β -actin band.

RV stimulation of ENA-78 gene transcription. A 753-bp fragment of the ENA-78 promoter was amplified by PCR from genomic DNA isolated from BEAS-2B cells by use of the following primers: ENA-702 (5'-TCTCAGTAAGCGGACTTACC-3') and ENA+51 (5'-GTGGAGGAGCGGAGATTGGG-3'). The resulting PCR product was cloned into the pGEM T-Easy vector (Promega) and was sequenced to verify that no mutations had occurred during the PCR. The 753-bp fragment of the ENA-78 promoter then was subcloned into pGL2-basic (Promega) to construct ENA-78-Luc. The ENA-78-Luc construct was cotransfected into BEAS-2B cells together with the cytomegalovirus (CMV) promoter pRL-CMV (Promega) with use of calcium phosphate [17]. Twenty-four hours after the transfection, the cells were infected with RV16 at an MOI of 1. Luciferase activity was measured 2 and 6 h after infection by use of the Dual Luciferase Reporter System (Promega). Transfections were repeated 3 times in triplicate, and the results were expressed as arbitrary units relative to Renilla luciferase expression.

Detection of ENA-78 in nasal secretions. To determine whether ENA-78 could be detected at sites of in vivo RV infection, we quantified the levels of ENA-78 in nasal aspirates obtained from 18 patients (aged 19–46 years). Subjects were part of a larger study of patients admitted to a medical emergency room because of acute severe symptomatic asthma (peak expiratory flow <65% predicted) [18]. Nasal aspirates were obtained at admission and also after 56 days [18, 19]. Eleven patients with RV detectable by reverse-transcription (RT)-PCR on admission were matched with 7 subjects in whom RV could not be detected. Five healthy volunteers were recruited from the staff of the hospital as a control group.

Nasal mucus was obtained with a suction pump after ad-

ministration of histamine (8 mg/mL) as a fine spray into each nostril, as described elsewhere [2, 9]. There was no indication that administration of histamine in this fashion with immediate sampling influences measurements of RV or cytokines [20]. Nasal aspirate (~1 mL) was mixed with 3 mL of virus transport medium (10% Hanks' balanced salt solution, 0.015 M sodium bicarbonate, 40 μ g/mL ampicillin, 200 μ g/mL amphotericin B, and 0.5% BSA) and transported on ice, and aliquots were made and frozen at -80°C until further analysis.

PCR for RV in nasal secretions. To examine the association of RV infection with ENA-78 in vivo, we studied patients with and without RV detectable by RT-PCR in nasal aspirates. The assay was done as described elsewhere [9] on nasal aspirates obtained at admission and after 56 days. In brief, 150 μ L of nasal aspirate sample was subjected to RNA extraction with a phenol-chloroform reagent (Tri Reagent; Sigma). Dried nucleic acid pellets were resuspended in 5 μ L diethyl pyrocarbonate water, and, after RT (BRL Superscript Reverse Transcriptase; Gibco), PCR was done as follows: 30 cycles of 45 s at 94°C, 45 s at 53°C, and 75 s at 72°C, followed by a 10-min incubation at 72°C. Samples were assayed in a total volume of 50 μ L, each containing 20 mM dCTP, dGTP, dATP, and dTTP, 50 pmol of each primer (OL-26 and OL-27), 5 U of *Taq* DNA polymerase (Gibco), and 50 mM MgCl₂. Product (10 μ L) was electrophoresed on 0.7% agarose/Tris acetate ethylenediaminetetraacetic acid gels, and a positive result was indicated by a band at the 380-bp position. Positive results were confirmed by Southern blot analysis by hybridization with an internal probe JWA1b labeled with [α -³²P]dCTP. All samples were analyzed in a blinded fashion and in duplicate and were confirmed as RV by restriction analysis [21].

Statistical analysis. Unless otherwise stated, values are expressed as mean \pm SD. Comparisons were made with the Wilcoxon signed rank sum test or Student's paired *t* test, as appropriate. Because baseline (recovery) nasal ENA-78 measurements varied widely among individuals, values measured on admission were expressed as percentages of values obtained at recovery and were compared by the Student's *t* test. The number of patients with decreases or increases in nasal ENA-78 after 56 days were compared by the χ^2 test. Statistical software Data Analysis and Data Analysis Plus (Microsoft Office 97, professional edition) were used for all calculations. *P* \leq .05 was considered to be statistically significant.

RESULTS

RV replication in BEAS-2B and primary cells. To determine whether RV16 replicated efficiently in the various cell lines and primary cells used in this study, the cells were infected with RV16 (MOI, 1). Supernatants were collected at various time points and

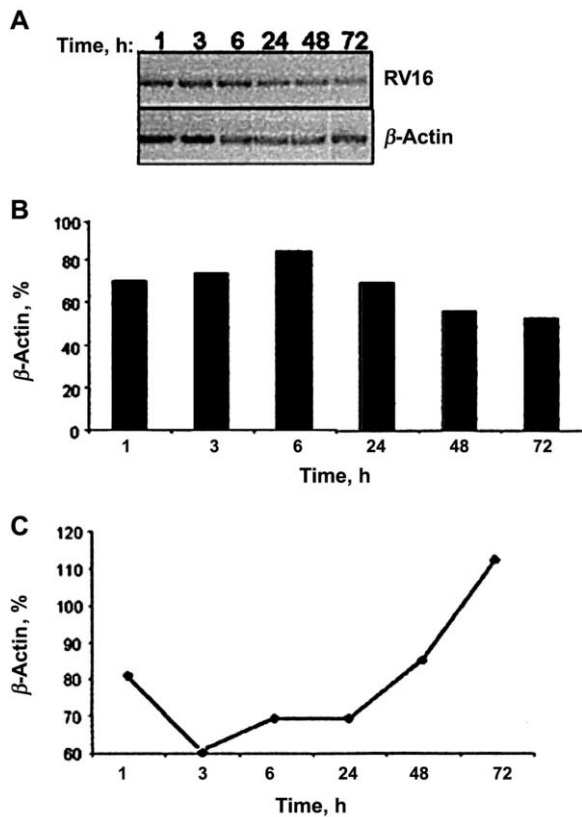


Figure 1. Rhinovirus serotype 16 (RV16) replication in BEAS-2B and primary cells. BEAS-2B cells were infected with RV16 (MOI, 1), and RNA was isolated from cells at various times after infection. RV16 levels were determined by reverse-transcription polymerase chain reaction (PCR), and resulting PCR products were electrophoresed on 1.5% agarose gels (A). Intensity of PCR product for RV16 was expressed as percentage of intensity of PCR product for β -actin (B). Similarly, RV16 replication was determined in primary epithelial cells (C).

were titrated on HeLa cell monolayers. In addition, at various times after infection, RNA was isolated from the cells and was subjected to RT-PCR for RV16 RNA with primers OL-26 and OL-27. RV16 replicated efficiently in BEAS-2B cells, but with negligible increases in TCID₅₀ in primary bronchial epithelial cells (data not shown). Use of RT-PCR to detect RV RNA demonstrated that the kinetics of replication differed in the 2 cell types (figure 1). In BEAS-2B cells, RV16 mRNA increased up to 6 h after infection, when it was maximal, and then decreased (figure 1A and 1B). In the primary cells, however, RV16 mRNA initially decreased from the time of infection and then steadily increased from ~10 h after infection (figure 1C).

RV stimulation of ENA-78 and IL-8 protein production in vitro. Infection of BEAS-2B cells with RV16 (MOI, 1) resulted in increased production of the CXC chemokines IL-8 and ENA-78 (figure 2). Enhanced chemokine production was dependent on the presence of live RV16, because incubation with UV-irradiated RV16 did not result in the same enhanced

chemokine production. The kinetics of chemokine production, as well as the absolute levels of chemokines produced, differed. IL-8 production after RV16 infection was elevated above that of mock-infected cells by 6 h after infection; however, a significant increase in ENA-78 production was observed only after 24 h. By 48 h after infection, both IL-8 and ENA-78 production were more than double the levels for mock-infected cells. ENA-78 production was increased from 6221 ± 595 pg/mL in mock-infected cells to $12,258 \pm 3193$ pg/mL in RV-infected cells ($P < .05$). IL-8 production was elevated from 1285 ± 163 pg/mL in mock-infected BEAS-2B cells to 3206 ± 529 pg/mL in infected cells at 48 h ($P < .05$).

Infection of primary bronchial epithelial cells with RV16 resulted in a maximal increase in ENA-78 after 48 h (figure 3A). Levels of ENA-78 production by primary epithelial cells were significantly lower than in BEAS-2B cells. ENA-78 production of 537 ± 312 pg/mL in mock-infected cells at 48 h was increased to 4723 ± 2124 pg/mL after RV16 infection ($P <$

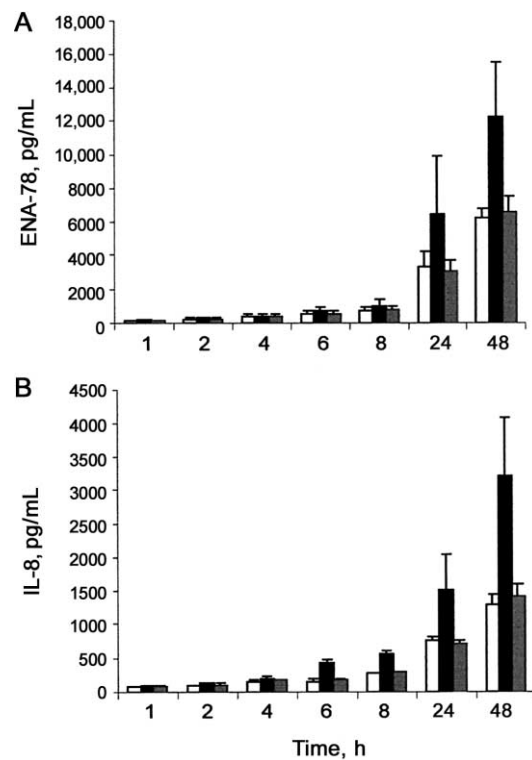


Figure 2. Effect of rhinovirus serotype 16 (RV16) infection (MOI, 1) of BEAS-2B cells on epithelial-neutrophil activating peptide-78 (ENA-78) (A) and interleukin (IL)-8 (B) production. Supernatants were collected from 1 to 48 h after infection, and chemokine concentrations were determined by ELISA. Open bars, chemokine concentration in absence of RV16 (mock infection); solid bars, chemokine concentration in presence of RV16; and shaded bars, chemokine concentrations in presence of UV-irradiated RV16. Moderate increases in IL-8 were noted after 8 h, whereas ENA-78 first increased 24 h after infection. Data are mean \pm SD of 4 independent experiments analyzed by paired *t* test.

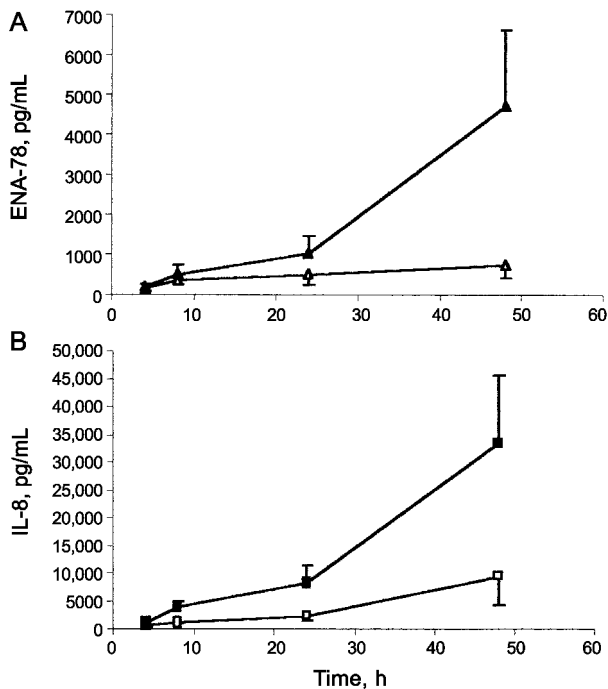


Figure 3. Effect of rhinovirus serotype 16 (RV16) infection (MOI, 1) of primary bronchial epithelial cells on epithelial-neutrophil activating peptide-78 (ENA-78) (A) and interleukin (IL)-8 (B) production. Supernatants were collected from 4 to 48 h after infection, and chemokine concentrations were determined by ELISA. *Open symbols*, chemokine concentration in absence of RV16 (mock infection); *solid symbols*, chemokine concentration in presence of RV16. Data are mean \pm SD of 3 independent experiments analyzed by paired *t* test.

.05). In comparison, increased production of IL-8 was detected early (8 h) after infection, and, by 48 h, production in RV-infected cells had increased to $33,421 \pm 12,221$ pg/mL versus 9437 ± 5231 pg/mL ($P < .05$) in mock-infected cells (figure 3B).

RV stimulation of ENA-78 and IL-8 mRNA in vitro. The effect of RV infection on the steady-state levels of ENA-78 and IL-8 mRNA was assessed by RT-PCR. Infection of the bronchial epithelial cell line BEAS-2B with RV16 at an MOI of 1 resulted in measurable increases in ENA-78 mRNA (data not shown). Induction of ENA-78 mRNA was observed at 6 h and again at 72 h after infection, and induction of IL-8 mRNA in BEAS-2B cells was observed after 6 h. Infection of primary bronchial epithelial cells also resulted in increases in both ENA-78 and IL-8 mRNA. Figure 4A and 4B show representative experiments for ENA-78 and IL-8, respectively. The maximal induction of each chemokine varied among primary cells obtained from different patients, but the trend remained consistent. ENA-78 mRNA was maximal 48 h after infection (figure 4A), whereas IL-8 mRNA was induced maximally by 24 h after infection (figure 4B).

RV stimulation of ENA-78 gene transcription. To determine whether RV infection had an effect on ENA-78 promoter

activity, a 753-bp fragment of the ENA-78 promoter was cloned upstream of the luciferase gene in pGL2-basic (ENA78-Luc) and used in transient transfection assays in BEAS-2B cells. A plasmid coding for Renilla luciferase under the control of the CMV promoter (pRL-CMV) was used as a control for transfection efficiency. Infection of BEAS-2B cells with RV16 at an MOI of 1 caused a 3-fold increase ($P = .05$) in ENA-78 promoter activity 2 h after infection, compared with that in uninfected cells (figure 5). Promoter activity remained elevated in the RV16-infected cells 24 h after infection. Cotransfection of pGL2-basic with pRL-CMV produced no significant luciferase activity (data not shown).

RV stimulation of ENA-78 protein in vivo. The characteristics of the subjects studied are shown in table 1. No differences in nasal cold symptoms or peak expiratory flow measurements were present. In the patient group with RV present, the mean concentration of ENA-78 in nasal aspirates was 5204 pg/mL (median, 2001 pg/mL; range, 123–21,650 pg/mL) at admission. It decreased to 1899 pg/mL (median, 399 pg/mL; range, 163–7000 pg/mL) after 56 days ($P = .03$). The mean

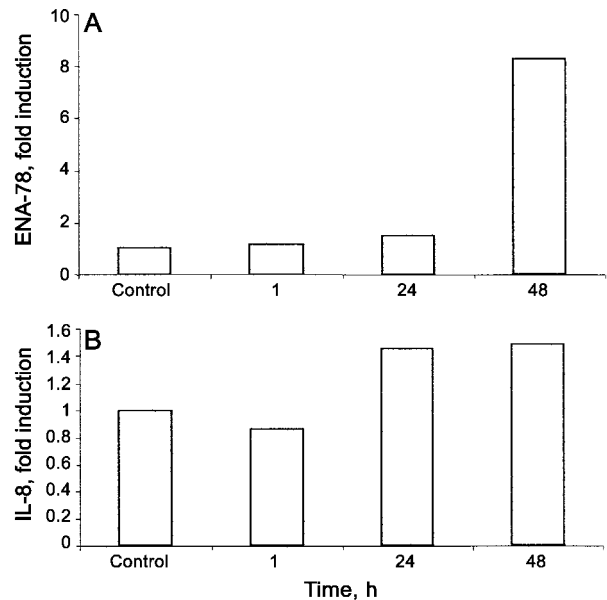


Figure 4. Analysis of epithelial-neutrophil activating peptide-78 (ENA-78) and interleukin (IL)-8 mRNA in primary human bronchial epithelial cells. Total RNA was isolated from primary human bronchial epithelial cells infected with rhinovirus serotype 16 (RV16; MOI, 1) and was analyzed by reverse-transcription polymerase chain reaction for ENA-78 (A) and IL-8 (B) expression. Fold induction of each chemokine is expressed relative to that of mock-infected control cells at each time point after normalization to β -actin expression. Representative results for 1 experiment are shown. Experiment was repeated 3 times with primary cells obtained from different patients, and, although the degree of induction varied between cells obtained from different patients, the trend was the same. Maximal induction of ENA-78 RNA occurred 48 h after infection, and IL-8 induction reached a maximum 24 h after infection. Control represents mock-infected cells.

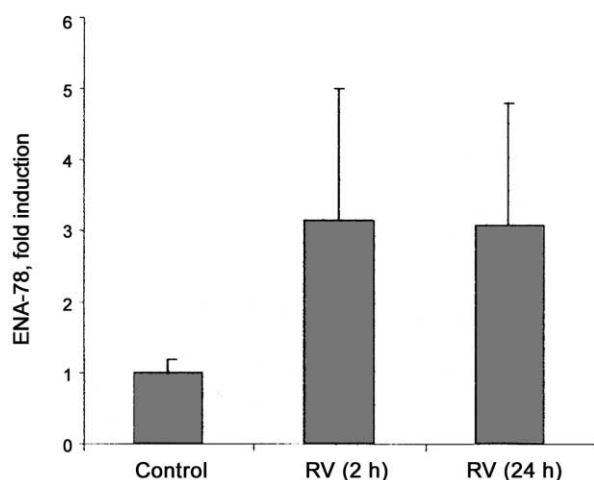


Figure 5. Analysis of epithelial-neutrophil activating peptide-78 (ENA-78) promoter activity: 753-bp fragment of ENA-78 promoter was cloned upstream of the luciferase gene in pGL2-basic and was transfected into BEAS-2B cells. Twenty-four hours after transfection, cells were infected with rhinovirus serotype 16 (RV16; MOI, 1), and luciferase activity was determined 2 and 24 h after infection. Results are normalized to Renilla luciferase expression and are represented as fold induction over luciferase expression in mock-infected cells. Infection with RV16 induced ENA-78 gene expression after 2 h ($P = .05$), which was still present 24 h after infection. Data are mean \pm SD of 3 independent experiments analyzed by paired t test.

level of ENA-78 in subjects without RV was 2439 pg/mL (median, 1400 pg/mL; range, 150–4750 pg/mL) at admission and 1456 pg/mL (median, 1100 pg/mL; range, 250–3050 pg/mL) after 56 days ($P = .4$). The ENA-78 ratio (ratio of measurements at admission to recovery—that is, after 56 days) was 4.1 ± 1.2 for the RV-positive group and 1.4 ± 0.5 for the RV-negative group, a difference that approached significance (figure 6; $P < .06$). In the group with nasal RV, 9 of 11 subjects had a decrease in ENA-78 after 56 days, compared with 3 of 7 without RV; 2 patients without RV showed unchanged levels ($P < .07$). The mean concentration of ENA-78 in nasal aspirates from the nonasthmatic control group was 530 pg/mL (median, 110 pg/mL; range, 200–1200 pg/mL; $P = .05$ vs. those with RV and $P = .03$ vs. those without RV on admission). There was no significant difference after 56 days between subjects with or without RV and control subjects ($P < .1$ and $P < .09$, respectively). IL-8 was also measured in nasal aspirates, but no significant differences were detected (data not shown).

DISCUSSION

Neutrophil influx is a recognized feature of viral infection involving the respiratory tract, and neutrophil recruitment probably is the result of chemokines released by epithelial cells. Chemokines such as ENA-78 may thus play a pivotal role in

the initiation of inflammatory events ultimately mediated by neutrophils. The present study demonstrates that RV infection of epithelium generates the release of ENA-78 protein in addition to IL-8, RV induces ENA-78 mRNA production in epithelial cells, ENA-78 gene activation is induced by RV infection, and ENA-78 is increased in vivo during RV infections. Our observations suggest that release of ENA-78 may represent a salient tissue response to RV infection of the airways.

Although IL-8 is currently perceived as the predominant CXC chemokine in the recruitment of neutrophils to sites of inflammation in a variety of diseases, the contribution of related family members is also likely to be of importance. ENA-78 is a 78-aa polypeptide member of the α -chemokine family that is homologous to IL-8, with which it shares a 22% sequence identity [22]. Although its role in human disease states has been examined in blood, synovial tissue, renal allograft recipients, and patients with inflammatory bowel disease and idiopathic pulmonary fibrosis [23–25], ENA-78 expression in response to RV infection has been examined in only 1 study [26]. In the latter study, BEAS-2B cells after infection with RV39 produced ENA-78, and production was reduced in response to an inhibitor of p38 kinase. Our studies examined responses to infection with a different serotype (RV16) in the same epithelial cell line (BEAS-2B) and in cultured primary epithelial cells, the cell type in which the virus initiates infection in vivo [27]. BEAS-2B cells were infected, on the evidence of both titration and RT-PCR for RV RNA, and infection could be demonstrated by RT-PCR in primary epithelial cells. Small changes in virus titration may be the result of lower numbers of infected cells (<10% in a recent study [28]); moreover, increased viral replication measured by titration techniques may have been detectable if a higher MOI was used to infect cells [28]. Because significant chemokine production was noted during pilot experiments at an MOI of 1, a higher MOI was not studied.

Production of ENA-78 protein was compared with that of IL-8 because of IL-8's known activities after RV infection [8, 9] and because ENA-78 may follow a different time course of production [29]. For example, in human alveolar macrophages, IL-8 rather than ENA-78 appears to be the predominant chemokine in supernatants obtained 24 h after lipopolysaccharide stimulation [30]. Our results demonstrate that significant amounts of ENA-78 are generated in response to RV and that ENA-78 protein and mRNA appear to be produced at a later stage than is IL-8 in epithelial cells (see figures 2–5). Prolonged activation of the ENA-78 gene by RV16 provides additional evidence for an ENA-78 response to RV that is different from that of IL-8. Taken together, these findings suggest that after the early release of IL-8, ENA-78 may augment and support ongoing neutrophil traffic from the vascular compartment to interstitial tissue spaces. Activation of neutrophils (initially recruited by IL-8) and their release of mediators through the

Table 1. Characteristics of 18 subjects with asthma and 5 control subjects in whom nasal epithelial-neutrophil activating peptide-78 (ENA-78) was measured.

Characteristic	Subjects with asthma		Control subjects (n = 5)
	RV present (n = 11)	RV absent (n = 7)	
Mean age, years	30.1	27.9	31.8
Cold symptoms, no. of cases	9	3 ^a	0
Peak expiratory flow, mean ± SD, % of predicted	50 ± 3.2	50.6 ± 4.3	ND
Atopy, no. of cases	8	5	1
Treatment	B ₂ (n = 11), inhaled CS (n = 7), T (n = 8), oral CS (n = 9), nasal CS (n = 3)	B ₂ (n = 7), inhaled CS (n = 4), T (n = 6), oral CS (n = 5), nasal CS (n = 2)	None

NOTE. B₂, B₂-agonist medication; CS, corticosteroids; ND, not done; T, theophylline. Peak expiratory flow measurements were done during acute asthma attack.

^a Nasal symptoms were not recorded for 1 patient.

actions of ENA-78, present in tissues at a later stage, may amplify the inflammatory cascade, culminating in kinin release, increases in vascular permeability, and a further influx of inflammatory cells, proteins, and mediators [5]. It should be recognized that the quantities of chemokine released may not reflect biological activities and proficiency to attract neutrophils, but to date no studies have been reported of a head-to-head comparison of the chemotactic potencies of IL-8 and ENA-78.

The reason(s) for the relative differences in chemokine production in primary cells and in the cell line BEAS-2B cannot be determined from our studies. Levels of ENA-78 released by infected BEAS-2B cells were moderately higher than those reported in the study by Griego et al. [26]. This may be a function of a different RV serotype (RV39 vs. RV16) or variations in culture conditions or infectious doses used [28]. Comparable studies of ENA-78 production by airway primary epithelial cells have not been reported. Measurements of IL-8 released by RV-infected BEAS-2B cells were similar to levels found in other studies [8, 9, 26]. However, we detected release of IL-8 from primary bronchial epithelial cells that was 2-4-fold higher than has been reported in a recent study [31]. Interestingly, levels of IL-8 comparable with those found in our study were produced by another airway epithelial cell line (A549 cells) infected with RV9 [32], again emphasizing the role of cell type and viral serotype (in addition to different MOIs) in variable chemokine responses. ENA-78 mRNA transcription and protein release both appeared to peak at 48 h; in addition, there was a more evident mRNA induction (8-fold over mock-infected; figure 4A) than protein release (2.5-fold over mock-infected; figure 3A). It may be that not all ENA-78 produced is destined for secretion, or that various isoforms of ENA-78 are expressed and only 1 is secreted. In the case of IL-8, we found that mRNA peaked at 24 h after RV infection of primary epithelial cells (1.5-fold increase over mock-infected; figure 4B), similar to levels reported in BEAS-2B cells [26]. The secretion of IL-8

protein peaked later at 48 h, as would be expected if newly synthesized protein was released (4.3-fold increase over mock-infected; figure 3B). Studies of mRNA stability and the presence and release of preformed mediators will clarify some discrepancies observed in this and other studies.

Specificity of cellular responses was assessed by means of mock infection and incubation with UV-irradiated RV16. Results from UV-irradiated controls were similar to those from mock infection, although some other investigators have reported slight increases above baseline [26, 33]. This may relate to UV-irradiation protocols and the presence of residual infectious virus; in experiments not detailed herein, we have found that a UV-irradiation time of <30 min can be associated with some viable RV detectable by titration. Other methods to inactivate RV, such as exposure to low pH, also may not be fully effective. However, it is possible that RV-intercellular adhesion molecule-1 interac-

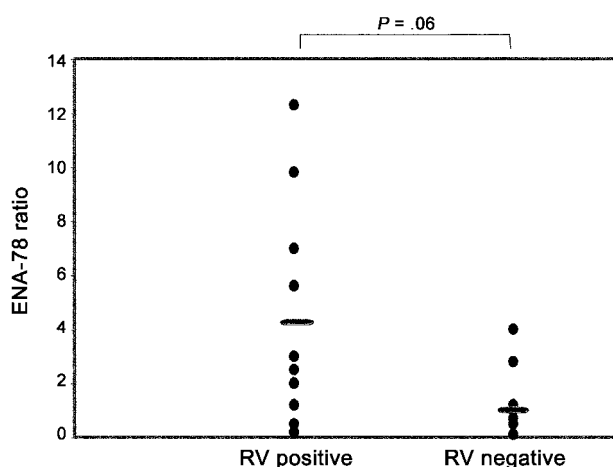


Figure 6. Comparison of epithelial-neutrophil activating peptide-78 (ENA-78) in subjects with and without nasal rhinovirus (RV), demonstrating increases in ENA-78 associated with RV infection. Ratios were calculated for values of ENA-78 obtained during acute asthma and at recovery. Horizontal bars indicate means. Statistical comparison was made by *t* test.

tions may induce signaling pathways independent of viral replication, and this requires further investigation.

The link between ENA-78 and RV in human infections has not been examined. We measured ENA-78 in a cross-sectional study of patients with acute asthma and healthy control subjects and found differences in nasal levels of ENA-78 that depended on the presence or absence of RV. In acute asthmatics, differences were noted between patients with and without nasal RV, and, in those with RV present, nasal levels of ENA-78 were significantly higher than at recovery after 56 days. Our observations suggest that RV induces release of ENA-78 in vivo and provides additional evidence for its role in human infections. We did not test for the presence of other viruses in the group without RV, and in some of them, viruses may have contributed to the moderate elevations in ENA-78 observed in some cases. However, significant differences between acute asthma and recovery were absent in the group without RV. The present studies did not investigate whether RV also induces ENA-78 from persons without asthma infected with RV. We were unable to address this question because the original study from which samples were generated did not include this group [18]. In contrast to community studies of children with asthma with RV infection [34] and during experimental RV16 infection [35], we could not detect differences in nasal IL-8 during infection. This may be related to age (adults) and timing of sample collection (later, in hospital) in our study.

The important role of respiratory virus infections as a cause of community morbidity and acute asthma is currently well recognized. Chemokines may play a central role in the initiation of inflammatory responses to viral infection and can be targeted for attempts to reduce or prevent subsequent detrimental events, particularly in patients with asthma. Our studies demonstrate an association between the ability of RV common cold viruses to induce ENA-78 production in vitro and their known ability to cause asthma exacerbations in vivo. These observations implicate ENA-78 in viral and allergic inflammation through its putative effects on neutrophils and also through additional possible actions on inflammatory gene expression [36] and angiogenesis [13] that may worsen airway inflammation. Characterization of ENA-78 regulation will provide insights into the complex biological responses that characterize RV infections and suggest strategies for their future therapeutic modulation.

Acknowledgments

We thank David Proud and Sebastian Johnston, for the gift of research materials and helpful discussions; Emma van Schalkwyk, for statistical advice; Zelda Williams and Wendy Lee, for expert technical and clinical assistance; and Jennie Saravanamuttu, for excellent secretarial assistance.

References

1. Nicholson KG, Kent J, Ireland DC. Respiratory viruses and exacerbations of asthma in adults. *BMJ* **1993**;307:982–6.
2. Johnston SL, Pattemore PK, Sanderson G, et al. Community study of role of viral infections in exacerbations of asthma in 9–11 year old children. *BMJ* **1995**;310:1225–9.
3. Seemungal T, Harper-Owen R, Bhowmik A, et al. Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **2001**;164:1618–23.
4. Winther B, Farr B, Turner RB, Hendley JO, Gwaltney JM Jr, Mygind N. Histopathologic examination and enumeration of polymorphonuclear leukocytes in the nasal mucosa during experimental rhinovirus colds. *Acta Otolaryngol Suppl* **1984**;413:19–24.
5. Naclerio RM, Proud D, Lichtenstein LM, et al. Kinins are generated during experimental rhinovirus colds. *J Infect Dis* **1988**;157:133–42.
6. Cheung D, Dick EC, Timmers MC, de Klerk EP, Spaan WJ, Sterk PJ. Rhinovirus inhalation causes long-lasting excessive airway narrowing in response to methacholine in asthmatic subjects in vivo. *Am J Respir Crit Care Med* **1995**;152:1490–6.
7. Jarjour NN, Gern JE, Kelly EA, Swenson CA, Dick CR, Busse WW. The effect of an experimental rhinovirus 16 infection on bronchial lavage neutrophils. *J Allergy Clin Immunol* **2000**;105:1169–77.
8. Subauste MC, Jacoby DB, Richards SM, Proud D. Infection of a human respiratory epithelial cell line with rhinovirus: induction of cytokine release and modulation of susceptibility to infection by cytokine exposure. *J Clin Invest* **1995**;96:549–57.
9. Papadopoulos NG, Bates PJ, Bardin PG, et al. Rhinoviruses infect the lower airways. *J Infect Dis* **2000**;181:1875–84.
10. Walz A, Burgener R, Car B, Baggiolini M, Kunkel SL, Strieter RM. Structure and neutrophil-activating properties of a novel inflammatory peptide (ENA-78) with homology to interleukin 8. *J Exp Med* **1991**;174:1355–62.
11. Bozic CR, Gerard NP, Gerard C. Receptor binding specificity and pulmonary gene expression of the neutrophil-activating peptide ENA-78. *Am J Respir Cell Mol Biol* **1996**;14:302–8.
12. Lukacs NW, Hogaboam CM, Kunkel SL, et al. Mast cells produce ENA-78, which can function as a potent neutrophil chemoattractant during allergic airway inflammation. *J Leukoc Biol* **1998**;63:746–51.
13. Keane MP, Belperio JA, Burdick MD, Lynch JP, Fishbein MC, Strieter RM. ENA-78 is an important angiogenic factor in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* **2001**;164:2239–42.
14. Zhu Z, Tang W, Ray A, et al. Rhinovirus stimulation of interleukin-6 in vivo and in vitro: evidence for nuclear factor kappa B–dependent transcriptional activation. *J Clin Invest* **1996**;97:421–30.
15. Papadopoulos NG, Stanciu LA, Papi A, Holgate ST, Johnston SL. A defective type 1 response to rhinovirus in atopic asthma. *Thorax* **2002**;57:328–32.
16. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* **1987**;162:156–9.
17. Chen CA, Okayama H. Calcium phosphate–mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques* **1988**;6:632–8.
18. Ferreira A, Williams Z, Donniger H, van Schalkwyk EM, Bardin PG. Rhinovirus is associated with severe asthma exacerbations and raised nasal interleukin-12. *Respiration* **2002**;69:136–42.
19. Einarsson O, Geba GP, Zhu Z, Landry M, Elias JA. Interleukin-11: stimulation in vivo and in vitro by respiratory viruses and induction of airways hyperresponsiveness. *J Clin Invest* **1996**;97:915–24.
20. Sanico AM, Stanisz AM, Gleeson TD, et al. Nerve growth factor expression and release in allergic inflammatory disease of the upper airways. *Am J Respir Crit Care Med* **2000**;161:1631–5.
21. Papadopoulos NG, Hunter J, Sanderson G, Meyer J, Johnston SL. Rhinovirus identification by *Bgl*I digestion of picornavirus RT-PCR amplicons. *J Virol Methods* **1999**;80:179–85.

22. Walz A, Strieter RM, Schnyder S. Neutrophil-activating peptide ENA-78. *Adv Exp Med Biol* **1993**; 351:129–37.
23. Koch AE, Kunkel SL, Harlow LA, et al. Epithelial neutrophil activating peptide-78: a novel chemotactic cytokine for neutrophils in arthritis. *J Clin Invest* **1994**; 94:1012–8.
24. Schmouder RL, Strieter RM, Walz A, Kunkel SL. Epithelial-derived neutrophil-activating factor-78 production in human renal tubule epithelial cells and in renal allograft rejection. *Transplantation* **1995**; 59:118–24.
25. Z'Graggen K, Walz A, Mazzucchelli L, Strieter RM, Mueller C. The C-X-C chemokine ENA-78 is preferentially expressed in intestinal epithelium in inflammatory bowel disease. *Gastroenterology* **1997**; 113:808–16.
26. Griego S, Weston C, Adams J, Tal-Singer R, Dillon S. Role of p38 mitogen-activated protein kinase in rhinovirus-induced cytokine production by bronchial epithelial cells. *J Immunol* **2000**; 165:5211–20.
27. Bardin PG, Johnston SL, Sanderson G, et al. Detection of rhinovirus infection of the nasal mucosa by oligonucleotide in situ hybridization. *Am J Respir Cell Mol Biol* **1994**; 10:207–13.
28. Mosser AG, Brockman-Schneider R, Amineva S, et al. Similar frequency of rhinovirus-infectible cells in upper and lower airway epithelium. *J Infect Dis* **2002**; 185:734–43.
29. Schnyder-Candrian S, Walz A. Neutrophil-activating protein ENA-78 and IL-8 exhibit different patterns of expression in lipopolysaccharide- and cytokine-stimulated human monocytes. *J Immunol* **1997**; 158:3888–94.
30. Goodman RB, Strieter RM, Frevert CW, et al. Quantitative comparison of C-X-C chemokines produced by endotoxin-stimulated human alveolar macrophages. *Am J Physiol* **1998**; 275:L87–95.
31. Konno S, Grindle KA, Lee WM, et al. Interferon-gamma enhances rhinovirus-induced RANTES secretion by airway epithelial cells. *Am J Respir Cell Mol Biol* **2002**; 26:594–601.
32. Johnston SL, Papi A, Bates PJ, Mastrorade JG, Monick MM, Hunninghake GW. Low grade rhinovirus infection induces a prolonged release of IL-8 in pulmonary epithelium. *J Immunol* **1998**; 160:6172–81.
33. Kaul P, Biagioli MC, Singh I, Turner RB. Rhinovirus-induced oxidative stress and interleukin-8 elaboration involves p47-phox but is independent of attachment to intercellular adhesion molecule-1 and viral replication. *J Infect Dis* **2000**; 181:1885–90.
34. Teran LM, Johnston SL, Schroder JM, Church MK, Holgate ST. Role of nasal interleukin-8 in neutrophil recruitment and activation in children with virus-induced asthma. *Am J Respir Crit Care Med* **1997**; 155:1362–6.
35. Grunberg K, Smits HH, Timmers MC, et al. Experimental rhinovirus 16 infection: effects on cell differentials and soluble markers in sputum in asthmatic subjects. *Am J Respir Crit Care Med* **1997**; 156:609–16.
36. Walz A, Schmutz P, Mueller C, Schnyder-Candrian S. Regulation and function of the CXC chemokine ENA-78 in monocytes and its role in disease. *J Leukoc Biol* **1997**; 62:604–11.