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J Immunol (2004) 172 (7): 4637–4645.
https://doi.org/10.4049/jimmunol.172.7.4637

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Human Rhinovirus Infection Induces Airway Epithelial Cell Production of Human β-Defensin 2 Both In Vitro and In Vivo[1]

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We hypothesized that airway epithelial cells, the primary site of human rhinovirus (HRV) infection, provide a link between the innate and specific immune response to HRV via production of human β-defensin (HBD)-2, a potent in vitro attractant and activator of immature dendritic cells. Infection of primary cultures of human epithelial cells with several HRV serotypes induced expression of HBD-2 mRNA and protein, indicating that HBD-2 production was independent of viral receptor usage or mechanisms of viral RNA internalization. Induction of HBD-2 was dependent upon viral replication and could be mimicked by transfection of cells with synthetic dsRNA, but was not dependent upon epithelial production of IL-1. Studies with stable epithelial cell lines expressing HBD-2 promoter constructs, as well as inhibitor studies in primary cells, both demonstrated that induction of HBD-2 involves activation of the transcription factor, NF-κB. Other transcription factors must also be activated by HRV infection, however, as expression of HBD-3 mRNA was also induced and there is no putative NF-κB recognition sequence in the promoter of this gene. HBD-2 showed no direct antiviral activity against HRV. In vivo infection of normal human subjects with HRV-16 induced expression of mRNA for HBD-2 in nasal epithelial scrapings. Increases in mRNA correlated with viral titer and with increased levels of HBD-2 protein in nasal lavages. This represents the first demonstration that HRV infection induces epithelial expression of HBD-2 both in vitro and in vivo, and supports the concept that HBD-2 may play a role in host defense to HRV infection. ***The Journal of Immunology*****, 2004, 172: 4637–4645.**

Human rhinovirus (HRV)[3] infections are the primary cause of the common cold (1), and are a major risk factor associated with exacerbations of asthma and chronic obstructive pulmonary disease (2–4). The mechanisms by which HRV infections induce or exacerbate airway symptoms, however, have not been delineated. In contrast to other common respiratory viruses, HRV infection results in no discernible changes in the integrity of the airway epithelium (5), implying that symptoms are unlikely to be attributable to cytotoxic effects of the virus.

In situ hybridization studies have established that the airway epithelial cell is the primary site of HRV infection in vivo (6, 7), and there is growing evidence that virally induced alterations in epithelial cell biology may contribute to disease pathogenesis. It has been shown that infection of cultured epithelial cells in vitro can induce the production of a variety of cytokines and chemokines, including IL-1, IL-6, IL-8, IL-11, GM-CSF, and RANTES (8–13). Furthermore, many of these cytokines also have been detected in airway secretions during in vivo rhinovirus infections (9, 10, 14, 15). Several of these cytokines are chemoattractants for, and/or activators of, a variety of inflammatory cell types. It is reasonable to hypothesize, therefore, that viral induction of epithelial cytokines can coordinate a local inflammatory response that may underlie symptom induction.

In addition to this proinflammatory response, however, HRV infection of epithelial cells also triggers additional immune responses to combat infection. For example, HRV infection, both in vitro and in vivo, induces epithelial expression of type 2 NO synthase (16). NO may contribute to the innate immune response to HRV infection because it inhibits both HRV-induced generation of several proinflammatory cytokines from epithelial cells (11, 17), as well as HRV replication (11). To date, however, little is known about factors that may link the innate and specific immune responses to HRV infection.

Defensins are cationic, antimicrobial peptides characterized structurally by the presence of six cysteine residues that form three intramolecular disulfide bonds. Based on the pairing of cysteines in these disulfide bridges, defensins are divided into the α- and β-defensin subfamilies (18). Although the human genome project implies there are over 20 potentially expressed genes, six members of the α-defensin family (19), and four β-defensins have been characterized thus far (20, 21). Human α-defensins 1–4 are major components of the human neutrophil azurophilic granules, whereas human α-defensins 5 and 6 are found in the secretory granules of intestinal Paneth cells (19). Human β-defensins (HBD)1–4 are commonly found in epithelial cells (20). Individual defensins display variable antimicrobial activities against bacteria and some show antiviral activities against enveloped viruses (22). HBDs also have been reported to impair viral infection under some conditions (23). Moreover, Human α-defensins 1–3 contribute to the ability of $CD8^+$ T cells to suppress HIV replication (24). It has recently been shown, however, that β-defensins may contribute to the immune response in other ways. HBD-2 has been shown to be chemotactic for immature dendritic cells and memory T cells via interactions with the chemokine receptor CCR6 (25, 26). Moreover,

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Received for publication October 8, 2003. Accepted for publication February 4, 2004.

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[1] This work was supported by Grant 43923 from the Canadian Institutes for Health Research, and by Grant HL 61011 from the National Institutes of Health. D.P. is the recipient of a Canada Research Chair in Inflammatory Airway Diseases.

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[3] Abbreviations used in this paper: HRV, human rhinovirus; HBD, human β-defensin; TLR, Toll-like receptor; BEGM, bronchial epithelial cell growth medium; CAPE, caffeic acid phenethyl ester; poly(U), polyuridylic acid; poly(I:C), polyinosinic:polycytidylic acid; $TCID_{50}$, 50% tissue culture-infective dose; IL-1Ra, IL-1R antagonist.

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HBD-2 has been reported to be able to induce Toll-like receptor 4 (TLR4)-dependent activation of immature dendritic cells (27).

The current studies were undertaken to test the hypothesis that HRV infection, in vitro and in vivo, will induce epithelial cell expression of HBD-2. This would suggest that HBD-2 may serve as an important link between innate and specific immunity to HRV infection.

Materials and Methods

Materials

The following reagents were purchased: Ham's F12 medium, Eagle's minimal essential medium, HBSS, penicillin-streptomycin-amphotericin B and L-glutamine (Medicorp, Montreal, Canada); TRIzol reagent, sodium pyruvate, nonessential amino acids, FBS and gentamicin (Invitrogen Life Technologies, Burlington, Ontario, Canada); bronchial epithelial cell growth medium (BEGM) (BioWhittaker, Walkersville, MD); Ab pairs for IL-1β and IL-1R antagonist (IL-1Ra), as well as recombinant proteins, were obtained from R&D Systems (Minneapolis, MN); LPS levels were assessed using a *Limulus* amoebocyte lysate assay (Charles River Endosafe, Charleston SC); caffeic acid phenethyl ester (CAPE) was obtained from Calbiochem-Novabiochem (San Diego, CA). The synthetic dsRNA, polyinosinic:polycytidylic acid (poly(I:C)), and ssRNA polyuridilic acid (poly(U)), as well as all other chemicals, were purchased from Sigma-Aldrich (St. Louis, MO).

Viruses and cell lines

HRV-16, HRV-14, HRV-2, HRV-1A, A549, and WI-38 cells were purchased from the American Type Culture Collection (Manassas, VA). Viral stock solutions of HRV-16 were generated by passage in WI-38 cells, as previously described (11). HRV-16 was further purified to remove ribosomes and soluble factors of WI-38 origin by centrifugation through sucrose, as described (28). Inactivation of HRV-16 was performed by exposure for 5 min to a Spectroline Model XX-15F high intensity short wavelength (254 nm) UV lamp at a distance of 5 cm. Inactivation was confirmed by the inability of stocks to replicate and cause cytopathic effects in WI-38 cells. For experiments using HRV-14, HRV-2, and HRV-1A, viral stocks were used directly as obtained from the supplier. Viral titers were assessed using WI-38 cells grown in 96-well plates, as previously described (11). Because bacterial LPS, at concentrations of 10 μg/ml, has been reported to induce HBD-2 expression (29), viral preparations were assayed for LPS. Levels of LPS were below limits of detection ($<$10 pg/ml) in all active and UV-treated viral preparations used in these studies.

Epithelial cell cultures

A549 cells were grown in Ham's F12 medium containing 5% FBS and antibiotics. Primary human adenoid epithelial cells were obtained by protease digestion of human tissue as previously described (30), and were grown on six-well culture plates in BEGM medium. Twenty-four hours before stimulation, primary epithelial cells were cultured in BEGM from which hydrocortisone had been withdrawn, and this hydrocortisone-free medium was used for all experimental exposures.

Viral infection of epithelial cells

Subconfluent cell monolayers were washed with HBSS and exposed to doses of 10^3–3×10^4 of 50% tissue culture-infective dose ($TCID_{50}$) units of HRV. This approximates to a multiplicity of infection ranging from 0.01 to 0.3. Cells were incubated at 34°C for the times indicated, at which point supernatants and total cellular RNA were harvested for subsequent analysis. To determine whether some HBD-2 may be retained intracellularly, cells were exposed to medium or HRV-16 for 48 h at 34°C. Supernatant medium was then removed and cells were lysed by scraping and sonication in 1 ml of medium. Lysates were then centrifuged and assayed for HBD-2 by ELISA. In experiments designed to inhibit activation of NF-κB, cells were infected with HRV-16 for 16 h before the addition of 15 μg/ml CAPE. A further 24-h incubation was then performed (for a total viral incubation of 40 h) before harvesting supernatants and cellular RNA. Assay of lactate dehydrogenase (Promega, Madison, WI) established that CAPE was not cytotoxic under these conditions. To evaluate the potential role of IL-1β as a mediator of HBD-2 production, cells were coincubated with HRV-16 and 400 ng/ml rIL-1Ra. Cells were also treated with IL-1β (100 pg/ml), in the presence or absence of IL-1Ra, to confirm that the concentration of IL-1Ra used was effective.

Epithelial cell transfection

Subconfluent primary epithelial monolayers were transfected with 1 μg/well of synthetic dsRNA poly(I:C) or ssRNA poly(U) for 5–6 h at 37°C using FuGene 6 (Roche, Laval, Canada). To assess effects of external stimulation, 1 μg of dsRNA also was added directly onto the monolayer. After the transfection period, wells were washed with HBSS and replaced with fresh BEGM medium (without hydrocortisone). After incubation for an additional 48 h, supernatants were harvested and RNA was isolated.

RNA extraction and real-time RT-PCR

Total cellular RNA was extracted from cultured epithelial cells with TRIzol (1 ml per 10 cm^2) as previously described (31). Yield of RNA was quantified based upon absorbance at 260 nm.

Gene expression for HBD-1, HBD-2, and HBD-3 were quantified using the Applied Biosystems Model 7900 Sequence Detector (Foster City, CA). To ensure there was no contribution of genomic DNA to amplification, samples were treated with DNase I (Ambion, Austin, TX) before use and probes were designed to cross introns. Input RNA (400 ng) was reverse transcribed to cDNA, followed by PCR amplification in the presence of specific forward and reverse primers and fluorescently labeled probes specific for each gene of interest. Analysis of the housekeeping gene GAPDH was performed on each sample using a primer and probe kit obtained from Applied Biosystems. For each gene of interest, we confirmed that efficiency of amplification was comparable to that of GAPDH (data not shown), permitting us to assess fold induction of genes using the formula $2^{-\Delta\Delta CT}$ as previously described (16).

Primers and probes for each defensin were as follows: HBD-1 forward primer 5′-CAGCAGTGGAGGGCAATGT-3′; reverse primer 5′-CCCTCTGTAACAGGTGCCTTG-3′; probe 5′-FAM-ATTCTGCCTGCCCGATCTTTACCAAAATT-TAMRA-3′. HBD-2 forward primer 5′-TCGTTCCTCTTCATATTCCTGATG-3′; reverse primer 5′-CCACTCTTAAGGCAGGTAACAGGAT-3′; probe 5′-FAM-CTTCCAGGTGTTTTTGG-MGB-3′. HBD-3 forward primer 5′-AGCTGCCTTCCAAAGGAGGA-3′; reverse primer 5′-TCGGCCACGCGTCG-3′; probe 5′-FAM-AGATCGGCAAGTGC-MGB-3′.

HBD-2 ELISA

Polyclonal antiserum to HBD-2 was produced in rabbits by injection of synthetic HBD-2 (Peptide Institute, Osaka, Japan) linked to keyhole limpet hemocyanin via carbodiimide coupling (32). Bleeds were tested using plate-bound HBD-2. Once high titer serum was obtained, an IgG fraction was produced by anion exchange chromatography. A portion of the IgG preparation was then biotinylated using Biotin-X-NHS (Calbiochem-Novabiochem). ELISAs were performed in microtiter plates (Immulon 4; Thermo Labsystems, Beverly, MA) coated with 1/2000 dilution of primary rabbit anti-human HBD-2 in 0.1 M carbonate buffer (pH 9.6), overnight at 4°C. Plates were washed three times with PBS/Tween 20 buffer (pH 7.4) and nonspecific binding sites were blocked by incubation for 30 min at room temperature with 1% rabbit serum diluted in PBS/Tween 80/1% BSA buffer. After washing, a 100 μl volume of standard or sample was added to each well as appropriate, and incubated at 37°C for 90 min. After repeated washing, 100 μl of 1/2000 dilution of biotinylated rabbit HBD-2 in PBS/Tween 80/1% BSA buffer was added to each well and incubated for 90 min at 37°C. Wells were again washed repeatedly and 100 μl per well of a 1 μg/ml solution of streptavidin-peroxidase was added and incubated for 30 min at room temperature. Positive samples were then detected by incubation with H_2O_2 and ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) as a substrate. Reactions were stopped by addition of 2 mM sodium azide and absorbance was read at 405 nm. All unknowns were assayed at three dilutions in duplicate. The sensitivity of the assay was 20 pg/ml HBD-2. There was no detectable cross-reaction (i.e., $<$0.01%) with either HBD-1 or HBD-3 (data not shown).

HBD-2 plasmid construction

A 1549-bp HBD-2 promoter region was amplified from human genomic DNA (Promega) using the following primers: 5′-GCTATGGTACCTAGCGTAAGCCATCATGC-3′ (sense) and 5′-GCATATGCTAGCTATGATGGCTGGGAGC-3′ (antisense). The 1549-bp promoter was cloned upstream of the inducible firefly luciferase gene in pGL3basic (Promega) via *Kpn*I and *Nhe*I restriction sites (underlined). A proximal 798-bp HBD-2 promoter construct was kindly provided by Dr. C. Bingle (University of Sheffield, Sheffield, U.K.). One of the NF-κB binding sites in the 798-bp HBD-2 promoter, located 237 bp upstream from the translational start site, was mutated using the QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX). The following primer sequence was designed to mutate the NF-κB site, 5′-TGGTCCCAAGAGCAGGAGGAACCTATTT

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TCTGGGGTTTCCTGAGT-3′ (mutated nucleic acids are underlined). All three constructs were digested with the appropriate restriction enzymes from pGL3 basic and subsequently ligated into pGL3basic.neo, a plasmid containing a neomycin cassette for antibiotic selection in cell culture (33). The pGL3basic.neo plasmid was kindly provided by Dr. R. Newton (University of Warwick, Coventry, U.K.).

Stable transfection

A transfection mixture containing 6 μg of each construct was incubated with 18 μl of FuGene 6 and 600 μl of Ham's F12 medium for 20 min at room temperature. Subconfluent monolayers of A549 cells were washed once with HBSS and incubated with the transfection mixture for 5 h at 37°C. The supernatant was aspirated and cells were fed with fresh A549 medium and left to incubate overnight at 37°C. A549 cells were fed every other day in medium containing 0.8 mg/ml geneticin (Invitrogen). After ~21 days, colonies were pooled and expanded. Cells were passaged into 6-well plates for subsequent stimulation in the absence of geneticin. Cells were grown to ~60% confluence and stimulated with 3×10^4 $TCID_{50}$U of HRV-16 for 48 h at 34°C. Luciferase activity was measured and data were expressed as fold increase over control.

In vivo experimental rhinovirus infections

The study protocol was approved by the Institutional Review Board of the Johns Hopkins Bayview Medical Center (Baltimore, MD), and informed consent was obtained from all subjects. Six healthy adults (five male and one female) with no detectable serum neutralizing Ab to HRV-16 were recruited. Subjects came to the laboratory on 5 consecutive days (days 0–4) and again on day 7. Following baseline nasal lavage and a nasal scraping, viral challenges were done by intranasal administration of rhinovirus type 16 (16). Volunteers were inoculated twice intranasally during a 1 h period with 0.25 ml/nostril delivered by pipette and three sprays per nostril (70 μl/spray) giving a total dose of ~1000 $TCID_{50}$ HRV-16.

Nasal scrapings were performed on day 0 and on day 2, with each scraping being performed in opposite nostrils. Nasal epithelial cells were collected from the inferior nasal turbinate by scraping the area with a curette (Rhinoprobe) and rinsing the probe in a sterile Eppendorf tube containing 200 μl of lactated Ringer solution. The cells were centrifuged at $300 \times g$ for 5 min at room temperature. The RNA was isolated using a Qiagen RNeasy kit (Valencia, CA) and used for real-time RT-PCR. As previously reported (16), cell differential analysis of nasal scrapings confirmed that ≥95% of cells were epithelial cells.

Nasal lavages were done with 10 ml of lactated Ringer solution in the morning on days 0–4 and day 7. The volume of recovered lavage fluids were recorded and fluids were divided into aliquots of 1 ml volume, and stored at −80°C for subsequent assay of HBD-2 protein and of viral titers by bioassay in WI-38 cells. Lavages were made 0.1% BSA before analysis. Infection was established by demonstrating an increase in serum neutralizing Ab of at least 4-fold and/or by the presence of virus in recovered secretions.

Subjects were given a previously described symptom questionnaire to fill out each morning and evening of all study days. As previously reported, criteria for a cold required a total symptom score of ≥5 over 4 days after challenge and/or the belief of the subject that a cold had occurred (14).

Antiviral effects of HBD-2

To determine whether HBD-2 had direct antiviral activity against HRV, four different purified stocks of HRV-16 were used. For each viral preparation, virus was incubated with an equal volume of medium, or with an equal volume of a solution of HBD-2 in medium. The final concentration of HBD-2 in these mixtures was 50 μg/ml (12 μM). After 7 h at 37°C, the level of infectious virus remaining was assessed using WI-38 cells.

Statistical analysis

For normally distributed data, appropriate one-way or repeat-measure ANOVA were used to assess significant differences, with post hoc analysis using Fisher's least significant difference tests. Alternatively, paired *t* tests were used. For data that were not normally distributed, analysis was performed using Kruskal-Wallis or Friedman ANOVA, followed by post hoc analysis using Wilcoxon matched-pairs signed-ranks test. Correlations were assessed using linear regression or Spearman rank analysis as appropriate. For all statistical tests, a p value ≤ 0.05 was assumed to be significant.

Results

HRV induces HBD-2 mRNA and protein release in a time-dependent fashion

HRV-16 infection of epithelial cells caused a significant ($p < 0.001$), time-dependent induction of mRNA expression for HBD-2. Significant increases in mRNA levels were not observed until 24 h after exposure to 10^4 $TCID_{50}$ U/ml HRV-16. Levels of mRNA continued to increase to a maximum of 7-fold ± 1.5 at 48 h after infection (Fig. 1). Gene induction was associated with a significant ($p < 0.01$) increase in levels of HBD-2 protein compared with medium control at 48 h after infection (Fig. 2*B*). Consistent with the late time course of mRNA production, virally induced protein release was not detected at times earlier than 48 h (data not shown).

Because it has recently been shown that HBD-2 can be packaged in lamellar bodies in human epidermis (34), we determined whether HBD-2 was also retained within epithelial cells in response to HRV infection. Lysates of epithelial cells exposed to medium alone for 48 h contained no detectable HBD-2, whereas lysates of HRV infected cells contained 31 ± 5 pg/ml ($n = 3$).

Induction of HBD-2 requires HRV-16 capable of replication

Given that that increases in mRNA and protein were not detected until relatively late after infection, we reasoned that generation of HBD-2 may depend upon pathways associated with viral replication. We, therefore, compared responses to HRV-16, which had been rendered incapable of replication by exposure to UV light, with those of fully functional virus. Intact HRV-16 induced increases in HBD-2 mRNA expression at both 24 and 48 h after infection. In each of four experiments, however, induction of HBD-2 mRNA was lower when UV-treated virus was used compared with intact virus (Fig. 2*A*). Indeed, UV-treated virus did not significantly increase mRNA levels compared with medium control. Striking differences between the two preparations were also noted when HBD-2 protein was measured at 48 h after infection. The UV-treated preparation generated HBD-2 levels that were significantly lower than those produced by the intact virus and which were not different from levels seen with medium alone (Fig. 2*B*). Thus, UV-treated virus was an ineffective stimulus for epithelial HBD-2 production.

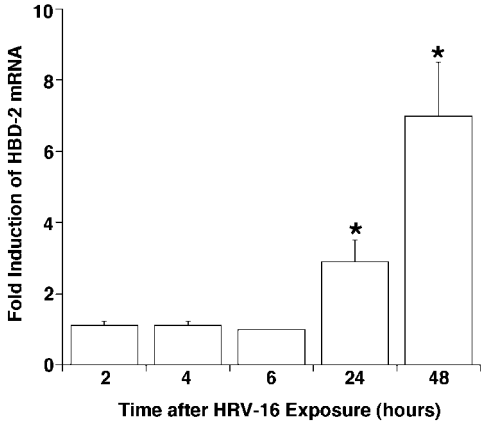

FIGURE 1. Time course of HBD-2 mRNA induction from HRV-16-infected human airway cells. Infection of primary human airway epithelial cells with 10^4 $TCID_{50}$ of HRV-16-induced HBD-2 mRNA in a time-dependant manner ($p < 0.001$ by ANOVA) is shown. Significant increases were seen at 24 and 48 h postinfection. Data represent mean ± SEM from eight experiments.

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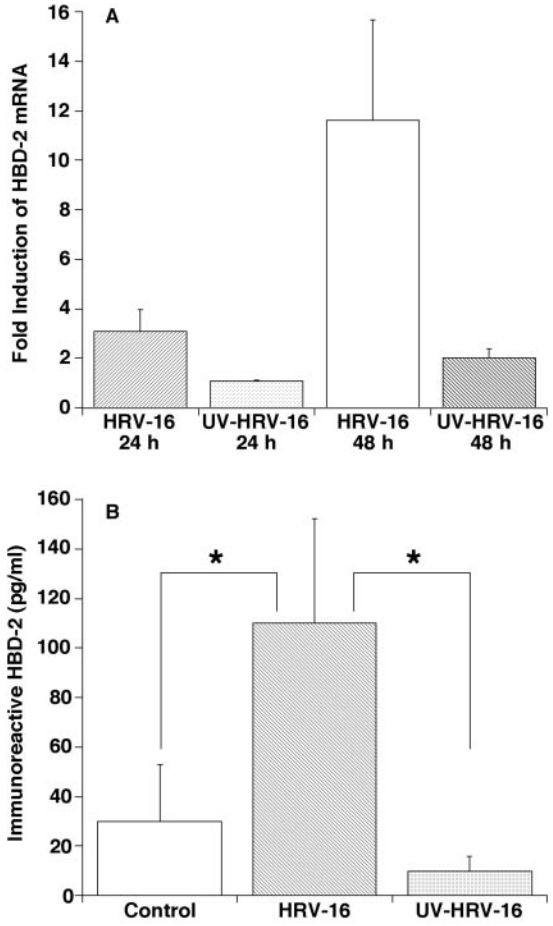

FIGURE 2. Expression of HBD-2 by human airway cells requires replicating rhinovirus. *A*, UV-inactivated HRV-16 does not induce mRNA for HBD-2 at either 24 or 48 h. Data represent mean ± SEM from four experiments. *B*, Release of HBD-2 protein was significantly reduced from epithelial cells exposed to UV-treated HRV-16 compared with those using intact virus ($p < 0.05$), and was not different from levels seen in cells exposed to medium control ($p = 0.65$). Data represent mean ± SEM from six experiments.

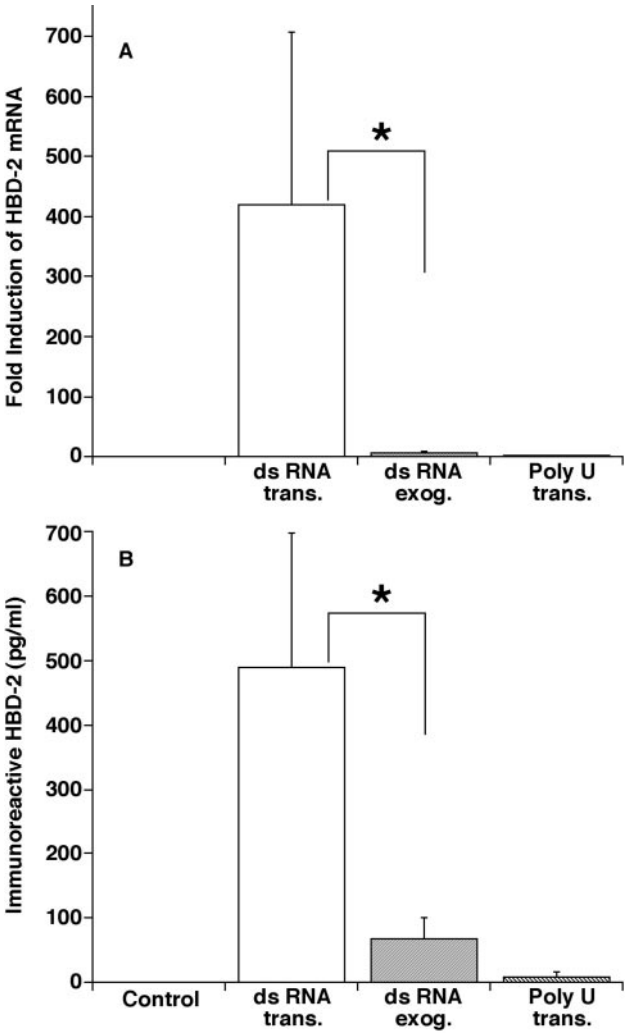

FIGURE 3. Effects of transfected dsRNA on HBD-2 production by human airway epithelial cells. Primary human airway epithelial cells transfected with 1.0 μg of dsRNA induced significant increases ($p < 0.01$) in mRNA (*A*) and protein (*B*) for HBD-2. Increases using transfected dsRNA were significantly greater ($p < 0.05$ in each case) than cells exposed to extracellular dsRNA. Transfection with ssRNA poly(U) was an ineffective stimulus for HBD-2 production. Data represent mean ± SEM from six experiments.

Double-stranded RNA is generated during the replication of HRV, and has been reported to be an important trigger of host antiviral responses and to induce the production of several cytokines (35–37). To evaluate the potential role of intracellular dsRNA in HRV induced HBD-2 production, we examined HBD-2 mRNA and protein production from cells 48 h after transfection with synthetic dsRNA, compared with responses with extracellular exposure to dsRNA and transfection with ssRNA poly(U). Transfected dsRNA induced striking and significant ($p < 0.01$) increases in HBD-2 mRNA expression, to a mean of over 400-fold that seen in cells exposed to medium alone (Fig. 3*A*). Similarly, protein levels in supernatants were also significantly ($p < 0.01$) increased (Fig. 3*B*). Although responses were seen in some experiments with exogenously applied dsRNA, this was inconsistent. Both mRNA and protein levels induced by exogenous dsRNA were significantly lower than those seen with transfected dsRNA ($p < 0.05$ in each case) and were not significantly different from levels induced by medium alone. Transfection of epithelial cells with ssRNA did not induce HBD-2 mRNA or protein.

HBD-2 induction by HRV-16 is not mediated via IL-1

It has been reported that IL-1β can induce epithelial expression of HBD-2 (29, 38). HRV infection of human airway epithelial cells is known to induce production of IL-1β (12), but also of IL-1Ra (39). We, therefore, examined whether viral induction of HBD-2 was secondary to IL-1 production. First, we measured levels of IL-1β and IL-1Ra in cell supernatants in which levels of HBD-2 were also assessed (Fig. 4*A*). In accordance with previous reports (12, 39), the mean level of IL-1β detected in supernatants 48 h after HRV-16 infection averaged 92 pg/ml, whereas the mean level of IL-1Ra was markedly higher (10,388 pg/ml). In six experiments, there was no significant correlation between concentrations of IL-1 and levels of HBD-2 produced ($r = 0.23$; $p = 0.65$). Indeed, the slope of the correlation was negative.

To further assess the role of IL-1, we also examined the effects of exogenous IL-1Ra on induction of HBD-2 mRNA expression 48 h after HRV-16 infection. Based on the manufacturer's potency data, a concentration of IL-1Ra of 400 ng/ml was selected as adequate to markedly inhibit effects of 100–200 pg/ml IL-1β. To confirm this, we also stimulated cells with 100 pg/ml IL-1β in the presence and absence of this level of IL-1Ra. Although there was some variability in the levels of virally induced gene expression

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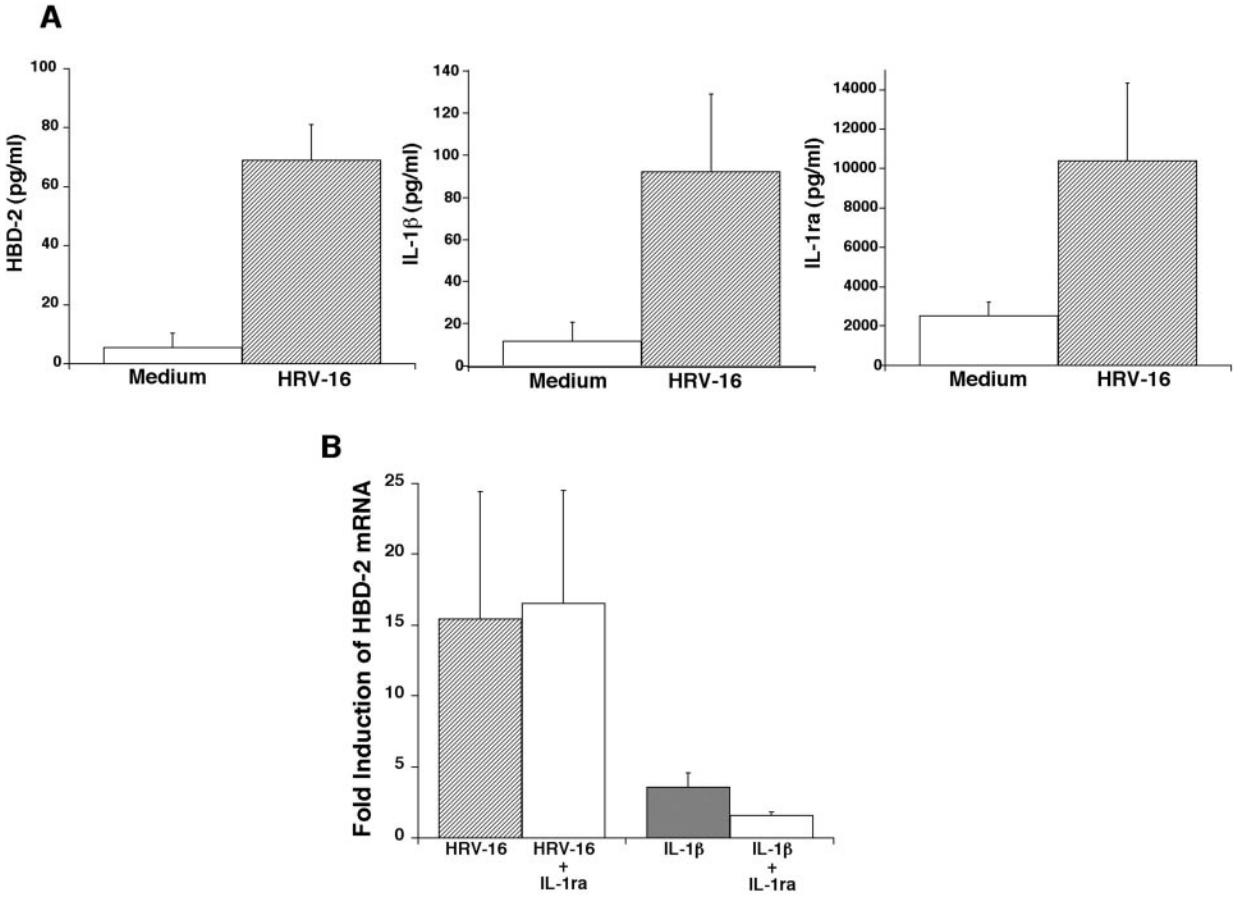

FIGURE 4. Induction of HBD-2 by HRV-16 infection is independent of IL-1β production. *A*, Levels of HBD-2, IL-1β and IL-1Ra in supernatants 48 h after exposure to medium alone or to HRV-16 are shown. Data represent mean ± SEM from six experiments. *B*, Exposure of cells to HRV-16 in the presence of 400 ng/ml IL-1Ra for 24 h does not inhibit virally induced increases in HBD-2 mRNA levels but inhibits the effects induced by 100 pg/ml IL-1β. Data are mean ± SEM from four experiments (for HRV-16) or three experiments (for IL-1β).

among experiments (Fig. 4*B*), there was no inhibitory effect of IL-1Ra on viral induction of HBD-2 mRNA in any experiment (15.4-fold ± 9 induction by HRV-16 vs 16.4-fold ± 8.1 for virus ± IL-1Ra, $n = 4$). By contrast, the effects of exogenously added IL-1β were modest and were inhibited by IL-1Ra (3.6-fold ± 1.1 induction by IL-1β alone compared with 1.6-fold ± 0.2 in the presence of IL-1Ra). Thus, induction of epithelial expression of HBD-2 in response to viral infection is not dependent upon IL-1 production.

Multiple serotypes of HRV induce HBD-2 production

The majority of rhinovirus serotypes use ICAM-1 as a cell surface receptor. The so-called minor group of serotypes, however, use members of the low density lipoprotein receptor family. To establish that HBD-2 induction was not a unique property of a single serotype of HRV, and was not dependent upon one type of viral receptor, we also examined the ability of HRV-14, (another major group rhinovirus) and of HRV-2 and HRV-1A (minor group rhinoviruses), to induce epithelial expression of HBD-2. Cells were exposed to 10^4 $TCID_{50}$ of each serotype and HBD-2 mRNA and protein were assessed after 48 h. In each of four experiments, all three serotypes also induced expression of HBD-2 (Fig. 5).

Effects of HRV-16 on gene expression for HBD-1 and HBD-3

Specific ELISAs for HBD-1 and HBD-3 were not available. Examination of the effects of HRV-16 infection on HBD-1 and HBD-3, therefore, were restricted to analysis of mRNA levels. HRV-16 infection did not increase epithelial expression of HBD-1 in any of four experiments. By contrast, HBD-3 mRNA levels increased by 8.2-fold ± 3.0 ($n = 6$) at 48 h after infection (data not shown).

HRV-16 effects on HBD-2 promoter activity and role of NF-κB

Stable lines of A549 cells expressing each of three promoter constructs were each infected with 3×10^4 $TCID_{50}$ of HRV-16. Cells expressing a promoter consisting of the 1549 bp upstream of the translational start site showed a 5-fold increase in luciferase activity 48 h after exposure to HRV-16 when compared with medium control. Truncation of this construct to the proximal 798 bp of the promoter resulted in no significant change in the induction of luciferase activity compared with the full-length construct. Because it has been shown previously that HRV infection of airway epithelial cells induces activation of NF-κB (10, 11, 40), and this transcription factor has been implicated in the IL-1 induced expression of HBD-2 in intestinal epithelial cells (41), we examined the effects of HRV-16 infection on activation of a variant of the 798 bp promoter in which the most proximal NF-κB recognition site was mutated. This mutation led to a significant reduction in luciferase activity ($p < 0.01$) compared with that seen with the nonmutated construct (Fig. 6).

We also examined the effects of CAPE, an inhibitor of NF-κB nuclear translocation, on endogenous HBD-2 gene expression induced by HRV infection. Treatment with CAPE led to a significant

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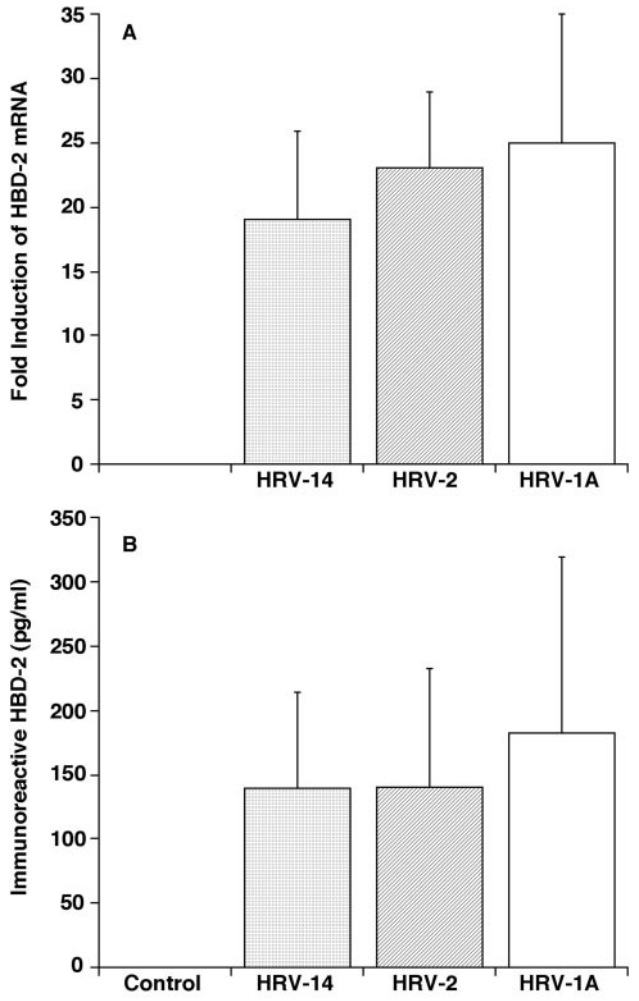

FIGURE 5. Induction of HBD-2 by multiple rhinovirus serotypes. Primary epithelial cells were infected with 10^4 $TCID_{50}$ of HRV-14 (a major group rhinovirus), HRV-2, or HRV-1A (both minor group rhinoviruses) and levels of HBD-2 mRNA (*A*) and protein (*B*) were assessed. Data represent mean ± SEM from four experiments. In every experiment, all virus strains increased both HBD-2 mRNA and protein.

($p < 0.05$) reduction in HRV-induced expression of HBD-2 mRNA, but did not completely inhibit gene induction (Fig. 7).

In vivo expression of HBD-2 during experimental HRV-16 infections

All six subjects used in the study became infected, as evidenced both by recovery of shed virus from nasal secretions and by appropriate increases in serum neutralizing Ab (Table I), and also developed symptomatic colds. Interestingly, detectable levels of HBD-2 protein were found in baseline (preinfection) lavages in four of the six subjects. Although symptom scores increased on day 1 after infection, HBD-2 levels in nasal secretions were not increased significantly ($p < 0.05$) until day 2 after viral exposure (Fig. 8). Concentrations of HBD-2 then remained elevated throughout the remainder of the study. When all time points were considered, there was a weak, but statistically significant ($p < 0.05$) correlation between HBD-2 levels and symptom scores (rho = 0.36). The epithelium appears to be a significant contributor to nasal HBD-2 production because mRNA levels in nasal epithelial scrapings obtained during the cold (day 2) were increased compared with levels preinfection in five of the six subjects (Table I). When data for day 2 were analyzed, fold increases

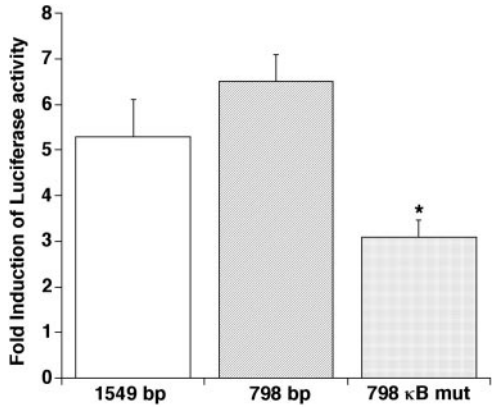

FIGURE 6. Luciferase activity from HRV-16 infected A549 cells stably transfected with various HBD-2 promoter constructs. Luciferase activity from cells exposed to 3×10^4 $TCID_{50}$ of HRV-16 was not different between cells expressing a 1549 bp promoter and those expressing a 798 bp promoter ($p = 0.14$). Mutation of the promixal NF-κB site (798 κB mut) lead to a significant ($p < 0.01$) reduction in promoter activity compared with the native 798 bp promoter. Data represent mean ± SEM from five experiments.

in HBD-2 mRNA levels correlated strongly both with fold increases in HBD-2 protein levels, and with increases in viral titer (rho = 0.77 in each case). Because of the small number of subjects, however, these data just failed to achieve statistical significance ($p = 0.08$ in each case).

HBD-2 does not directly inactivate HRV-16

Incubation of four different purified preparations of HRV-16 with 12 μM HBD-2 for 7 h did not lead to significant viral inactivation ($p = 0.64$). The log of viral titers (mean ± SEM) after incubation with medium alone was 3.3 ± 0.15 compared with 3.25 ± 0.09 upon incubation with HBD-2 (data not shown).

Discussion

HRV infections elicit Ag-specific humoral and cellular responses, but these are not detected until late in the resolution of symptomatic infections. Thus, both serotype-specific neutralizing Abs in

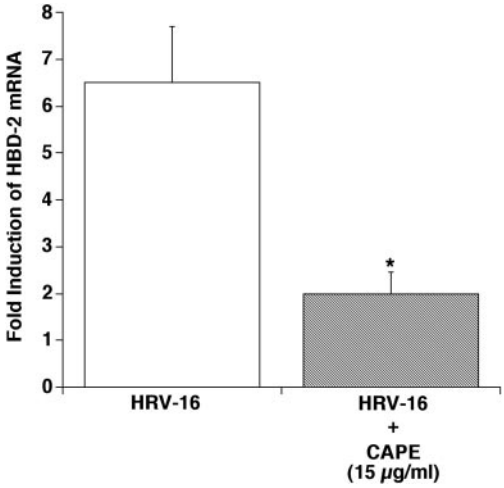

FIGURE 7. Effect of CAPE, a pharmacological inhibitor of NF-κB nuclear translocation, on HRV-16 induction of endogeneous HBD-2 gene expression by human airway epithelial cells. Treatment with CAPE (15 μg/ml) significantly ($p < 0.05$) inhibited induction of HBD-2 mRNA levels by infection with 10^4 $TCID_{50}$ of HRV-16. Data represent mean ± SEM from four experiments.

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Table I. *Responses to in vivo experimental HRV infection*

Subject	Ab Titer		Day 2 Response		
	Before Cold	After Cold	HBD-2 mRNA (fold increase)	HBD-2 Protein (fold increase)	Viral Titer (log units)
1	0	4	2.2	3.4	1.4
2	0	4	1.3	2.0	2.6
3	0	16	1.0	1.1	2.5
4	0	8	2.9	2.4	4.4
5	0	32	5.5	7.6	4.9
6	0	32	3.3	2.2	4.1

nasal secretions and serum, and virus-specific T cell responses are not detectable until ~2 wk postinfection (42–45). Given the time frame of these responses, it is generally assumed that the innate immune response is largely responsible for limiting symptomatic responses to HRV infections, whereas the specific cellular and humoral immune responses contribute primarily to final viral clearance and to protection from subsequent infection with the same viral serotype (46). Little is known, however, regarding links between the innate and specific immune responses to HRV infection.

The symptomatic response to HRV infection is largely restricted to the airway mucosal compartment, and the epithelial cell is the primary site of HRV infection in the airway. Growing evidence suggests that viral modulation of epithelial function may play an important role in the host immune response. The current study provides further support for this concept by demonstrating that HRV infection of epithelial cells, both in vitro and in vivo, induces production of HBD-2, a peptide that could play a central role in initiating the specific immune response, both via recruitment and activation of immature dendritic cells to initiate Ag presentation and via recruitment of memory T cells (25–27).

Induction of HBD-2 mRNA expression was not observed before 24 h after viral exposure, with protein not being detected until the 48 h time point. Although protein was secreted from epithelial cells, some protein remained intracellular. Given that a complete replication cycle for Picornaviridae, such as HRV, requires some 10 h post infection (47), we reasoned that induction of HBD-2 may depend upon the build up of cellular levels of products generated during the replication cycle. Consistent with this concept, HRV-16 that had been rendered replication deficient by brief exposure to UV light failed to induce HBD-2 mRNA or protein expression. Among potential viral replication products that could be responsible for HBD-2 generation, an obvious candidate is dsRNA, which has been shown to not only be a potent stimulus for host antiviral response pathways but also for cytokine production (35–37). Most studies have attempted to mimic effects of intracellular dsRNA generated during viral replication by adding dsRNA to the extracellular mileau on the assumption that it is internalized into cells. The recent demonstration, however, that extracellular dsRNA serves as a ligand for cell surface TLR3 clearly complicates interpretation of data using this approach (48). In the current studies, therefore, we performed pilot dosing experiments to establish a dose of dsRNA that induced little or no HBD-2 induction when applied extracellularly and then examined responses when cells were transfected with this dose. Our data demonstrate that 1 μg of dsRNA was an extremely effective stimulus for HBD-2 induction, stimulating a mean 400-fold increase in mRNA levels, when transfected into epithelial cells. The same dose applied outside of the cell was relatively ineffective. Indeed, in agreement with a recent report (37), we found that 30–100 μg of extracellular dsRNA were required to induce a response comparable to that seen with transfection of 1.0 μg (data not shown). Induction of HBD-2 by intracellular dsRNA was not an artifact of transfection because no response was observed when ssRNA was transfected. These data suggest that intracellular dsRNA, such as would be produced during viral infection, is a much more effective stimulus for HBD-2 induction than stimulation of cell surface TLR3. Alternatively, the affinity of cell surface TLR3 for dsRNA may be relatively low. We cannot definitively rule out that transfected dsRNA may interact with intracellular TLR3, but are aware of no data supporting the existence of intracellular TLR3 in epithelial cells. Although our data are consistent with a role of viral dsRNA in HBD-2 generation, additional studies will be needed to confirm that this is important in HRV-induced HBD-2 expression, and to examine potential pathways involved. For example, whereas activation of dsRNA activated protein kinase R has been implicated in several dsRNA or virally induced responses (49, 50), it has recently been shown that dsRNA-induced cytokine production from epithelial cells can also occur via an as yet to be delineated protein kinase R-independent pathway (51).

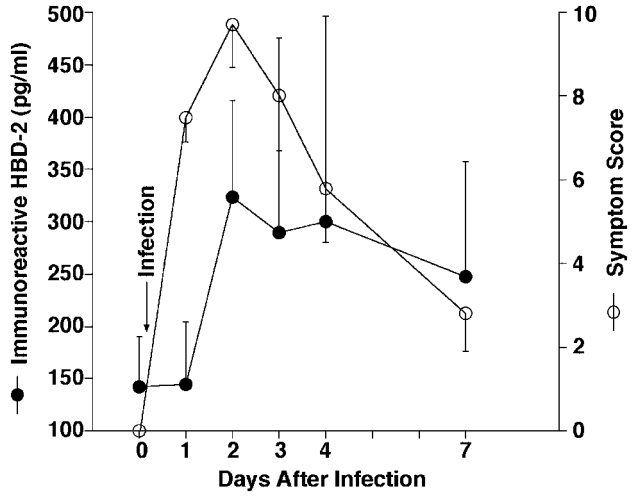

FIGURE 8. Effects of in vivo experimental HRV-16 infection on HBD-2 levels in airway secretions recovered by lavage. Increases in HBD-2 levels related to symptom scores. HBD-2 levels were first significantly elevated on day 2. Data are mean ± SEM from six subjects.

Because of the slow induction of HBD-2, it was possible that gene expression induced by HRV infection may be secondary to the generation of another epithelial cytokine. One likely candidate was IL-1β, which is produced by HRV infected epithelial cells, and is a stimulus for epithelial expression of HBD-2. Our data, however, do not support a role for IL-1 in virally induced expression of HBD-2. Not only did levels of IL-1β released not correlate with HBD-2 production, but addition of IL-1Ra at a concentration adequate to block levels of IL-1β detected had no effect on HBD-2 induced by HRV-16 infection.

The ability of members of both the major and minor groups of rhinoviruses to induce epithelial expression of HBD-2 indicates that HBD-2 production is a general response to infection with many HRV serotypes. Moreover, members of the major group of rhinoviruses use ICAM-1 as a cell surface receptor (52), whereas minor group rhinoviruses use members of the low density lipoprotein receptor family (53). In addition, there are differences in viral entry mechanisms not only between the two rhinovirus groups, but also among individual serotypes. HRV-2 uses clathrin-mediated endocytosis and late endosomes to achieve internalization of viral RNA (54, 55). Among the major group members, uncoating of viral RNA can occur simply as a result of binding of HRV-14 to

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ICAM-1, whereas this is not observed for other serotypes, such as HRV-16 (56, 57). Thus, the ability of all of the viral serotypes tested in this study to stimulate epithelial HBD-2 production implies that this induction is independent of both receptor subtype and viral RNA entry pathways, and thereby provides further support for the role of viral replication products in HBD-2 induction.

To determine whether HRV infection could also induce other members of the β-defensin family, we also examined mRNA expression for HBD-1 and HBD-3. We were unable to assess protein secretion for these peptides as specific ELISA were not available to us. HRV-16 infection clearly increased mRNA levels for HBD-3. By contrast, no effect was seen on expression of HBD-1 mRNA. This latter observation is consistent with previous reports indicating that HBD-1 is constitutively expressed in epithelial cells and is not induced by other proinflammatory stimuli (38, 41). By contrast, HBD-3 is known to be an inducible product in epithelial cells (58, 59). Overall, our data are also consistent with a very recent report that found HRV infection of cultured human epithelial cells induced expression of mRNA for HBD-2 and HBD-3, but not HBD-1 (60). This latter report, however, was unable to assess defensin protein expression, and did not expand on these observations.

To begin to assess the transcriptional regulation of HBD-2 in response to HRV-16 infection, we performed studies with promoter constructs. Initially, experiments were conducted using transient transfection of epithelial cell lines with these constructs, but we failed to detect induction of luciferase activity in response to HRV-16 infection with any of these constructs. Given that transient transfection introduces constructs into only a portion of the cells, and recent studies demonstrating that HRV infection also infects only a fraction of epithelial cells (61), we reasoned that the lack of measurable responses reflected a lack of sensitivity. We therefore generated stable cell lines expressing each of the constructs in question. Using this approach we were able to detect activation of a full-length 1549 bp construct. Truncation to 798 bp, which deleted numerous transcription factor recognition sites, including several putative sites for AP-1, AP-2, cEPB/β, and c-*rel*, had no significant effects on promoter activity, indicating that the 798 bp promoter contained all of the necessary transcription sites for gene activation in response to HRV infection.

The role of NF-κB activation in the induction of HBD-2 is somewhat controversial. Blockade of NF-κB activation inhibited IL-1α induced induction of HBD-2 in intestinal epithelial cell lines (37), but did not effect bacterial induction of HBD-2 expression in gingival epithelial cells (59). Mutation of the more proximal of two NF-κB sites in the 798 bp HBD-2 promoter construct, significantly reduced HRV-16 induced luciferase activity in respiratory epithelial cells. In addition, inhibition of translocation of NF-κB to the nucleus using CAPE inhibited increases in endogenous gene activation in primary epithelial cells. It should be noted, however, that the mutation of the proximal NF-κB site only partially inhibited HBD-2 promoter activity. Similarly, CAPE, an inhibitor of NF-κB translocation, did not completely inhibit mRNA induction. Moreover, as previously noted, HRV infection is also capable of inducing expression of HBD-3, and the promoter for HBD-3 contains no NF-κB recognition sites (59). Thus, whereas our data clearly implicate NF-κB activation in HRV-16-induced gene activation of HBD-2, it seems likely that other transcription factors contribute to HRV-induced β-defensin gene activation, and further studies are needed to delineate these pathways.

To ensure that HRV-induced epithelial production of HBD-2 was not just a feature of cell culture models, we experimentally infected healthy, seronegative volunteers with HRV-16. Our data provide the first demonstration that levels of HBD-2 are increased in airway secretions during symptomatic HRV infections. The relationship between HBD-2 levels and symptom scores was statistically significant but weak, with HBD-2 levels increasing more slowly than symptoms and remaining elevated as symptom scores declined. The fact that HBD-2 protein levels were not elevated until 48 h after infection was consistent with the time course observed in vitro. Increases in HBD-2 levels in nasal secretions at this time point correlated with increases in expression of HBD-2 mRNA in nasal epithelial scrapings obtained at the same time point, strongly supporting the epithelial origin of the protein in nasal secretions. Finally, the strong correlation between increases in mRNA and viral titers at the peak of the cold is also consistent with our in vitro data indicating that viral replication products induce HBD-2 expression.

Mean peak levels of HBD-2 protein detected in nasal lavages were in the order of 500 pg/ml. It is difficult to accurately extrapolate this to concentrations in undiluted nasal secretions, but it would not be unreasonable to suggest that the 10 ml lavage dilutes by ~50- to 100-fold. Thus, concentrations in undiluted secretions would be in the order or 25–50 ng/ml (5–10 nM). These levels are in reasonable agreement with levels found in airway secretions by others (62), but are well below the micromolar levels of defensins reportedly needed to directly inactivate enveloped viruses (22, 24). Moreover, in our current studies, even 12 μM HBD-2 was without effect on preparations of HRV-16, a nonenveloped virus. Thus, HBD-2 is unlikely to play a direct antiviral role during HRV infections. It is of interest, however, that the levels of HBD-2 detected in nasal secretions are well within the range reported to be chemoattractant for immature dendritic cells and memory T cells (25), and thus may contribute to such a chemotactic response in vivo.

In summary, we have provided the first demonstration that HRV infection induces production of HBD-2 mRNA and protein both in vitro and in vivo. Induction of HBD-2 is not dependent upon rhinovirus receptor subtype or internalization route, but requires replicating virus, and can be mimicked by intracellular dsRNA, suggesting that generation of viral dsRNA may be an important step in HBD-2 induction. Viral induction of HBD-2 is not mediated by epithelial production of IL-1β. Induction of HBD-2 gene expression is dependent upon activation of NF-κB but may also involve other, as yet unidentified, transcription factors. HBD-2 has no direct antiviral activity against HRV. Levels of HBD-2 detected in vivo, however, are in the range reported to be capable of recruiting and activating immature dendritic cells, and recruiting memory T cells in vitro. We speculate, therefore that HBD-2 exerts similar effects during HRV infections in vivo, and thereby, may play an important role in linking innate and specific immunity against HRV infections.

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