Synergism between Rhinovirus Infection and Oxidant Pollutant Exposure Enhances Airway Epithelial Cell Cytokine Production

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Of the several factors believed to exacerbate asthmatic symptoms, air pollution and viral infections are considered to be particularly important. Although evidence indicates that each of these respiratory insults individually can increase asthma severity in susceptible individuals, we know little about the extent to which exposure to environmental oxidant pollutants can influence the course of respiratory viral infection and its associated inflammation. To investigate the interaction of these two stimuli within their common epithelial cell targets in the upper and lower respiratory tracks, we infected primary human nasal epithelial cells and cells of the BEAS-2B line grown at the air–liquid interface with human rhinovirus type 16 (RV16) and exposed them to NO_2 (2.0 ppm) or O_3 (0.2 ppm) for 3 hr. Independently, RV16, NO_2, and O_3 rapidly increased release of the inflammatory cytokine interleukin-8 through oxidant-dependent mechanisms. The combined effect of RV16 and oxidant ranged from 42% to 250% greater than additive for NO_2 and from 41% to 67% for O_3. We abrogated these effects by treating the cells with the antioxidant *N*-acetylcysteine. Surface expression of intercellular adhesion molecule 1 (ICAM-1) underwent additive enhancement in response to combined stimulation. These data indicate that oxidant pollutants can amplify the generation of proinflammatory cytokines by RV16-infected cells and suggest that virus-induced inflammation in upper and lower airways may be exacerbated by concurrent exposure to ambient levels of oxidants commonly encountered the indoor and outdoor environments. *Key words*: **bronchial epithelium, ICAM-1, IL-8, nasal epithelium, nitrogen dioxide, oxidant stress, ozone.** *Environ Health Perspect* **110:665–670 (2002). [Online 28 May 2002]**
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We have recognized for many years that environmental agents can modify the course of respiratory viral infections, and ambient levels of these agents have been proposed to play a role in modulating disease severity and health outcome in exposed individuals. Early studies that investigated this relationship demonstrated that the interactive effect depended on both the infective agent used and the nature and extent of environmental exposure. Several of these studies made the observation that exposure of animals and human subjects to oxidant pollutants increased viral infective potential as well as the magnitude of the resulting virus infection (*1–3*). As recent evidence continues to support the hypothesis that viral infections can trigger exacerbations of asthma (*4–6*), renewed interest has developed in the modulating effect that environmental pollutants can have on the infective process and on the severity of resulting disease symptoms.

In their unique location at the interface between the air space and the submucosal regions, the epithelial cells of the upper and lower respiratory tract serve as common targets for airborne pollutants and several viral pathogens, including the rhinoviruses (*7–9*). A pathophysiologic consequence shared by rhinovirus infection and exposure to oxidant pollutants is an inflammatory response involving the influx of neutrophils and other cells into the airway tissues and air spaces. Although the mechanisms through which an upper respiratory infection might mediate lower airway inflammatory processes are currently unresolved, the potential importance of this inflammation in the precipitation of asthmatic episodes remains a focus of great interest (*10,11*). Respiratory tract inflammation is mediated largely by the release of proinflammatory mediators from epithelial cells (*12,13*) and is often linked to oxidant-responsive pathways (*14,15*). Interactions between biologic and chemical stimuli that may share activation pathways have been well established in a variety of cell types and can be either additive or synergistic in nature. These include tumor necrosis factor α (TNF_α), interleukin 1 (IL-1), interferon γ (IFN_γ), phorbol ester, calcium ionophore, and respiratory syncytial virus (*16–19*), all of which can activate "oxidant-sensitive" transcription factors. Despite its implications for contributing to asthma disease severity, essentially nothing is known about the extent to which rhinovirus-induced inflammation can be modified by the exposure of infected airway epithelium to the commonly encountered oxidant pollutants nitrogen dioxide (NO_2) and ozone (O_3).

We undertook the present study to investigate the interactive effects of human rhinovirus type 16 (RV16) and the oxidants NO_2 and O_3 on markers of proinflammatory activity in human bronchial and nasal epithelial cells. Our results demonstrate that the expression and release of IL-8 and the surface expression of intercellular adhesion molecule 1 (ICAM-1) by infected airway epithelial cells are markedly increased by subsequent exposure of the cells to either of the pollutant gases at moderate ambient levels. These results provide evidence for a mechanism through which environmental oxidant pollutants may exacerbate the pathology of respiratory viral infections *in vivo*.

Materials and Methods

Cell culture. We obtained cells of the BEAS-2B line from the American Type Culture Collection (ATCC; Bethesda, MD) and used them below passage 45. We expanded cultures by growing them on 100-mm plastic dishes in Ham's F-12 medium (Biofluids, Rockville, MD) containing insulin (5 µg/mL), hydrocortisone (7.2 ng/mL), epidermal growth factor (12.5 ng/mL), endothelial cell growth supplement (3.75 µg/mL; Gibco, Gaithersburg, MD); triiodothyronine (6.5 ng/mL), cholera toxin (10 ng/mL; Sigma, St. Louis, MO), glutamine (2 mM), retinoic acid (0.1 ng/mL), trace elements (30 nM $NaSeO_3$, 1 nM $MnCl_2 \cdot 4H_2O$, 0.5 µM $Na_2SiO_3 \cdot 9H_2O$, 1 nM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 5 nM NH_4VO_3, 1 nM $NiSO_4 \cdot 6H_2O$, 0.5 nM $SnCl_2 \cdot 2H_2O$), penicillin (100 U/mL), streptomycin (100 µg/mL), fungizone (0.25 µg/mL; Biofluids). We passaged cells to 24-well Falcon filter inserts (0.4 µm pore size; Becton Dickinson, Franklin Lakes, NJ) coated with Vitrogen 100 (1:3 in 60% ethanol; Celtrix, Palo Alto, CA) and grew them to confluence with the same medium above and below. When the cultures became confluent, we increased the concentration of Ca^{2+} in the medium above and below the cells to 1.2 mM for at least 18 hr. We

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removed medium from the apical surfaces 1–4 days before study.

Consistent with procedures approved by the Committee on Human Research of The Johns Hopkins Bloomberg School of Hygiene and Public Health, we obtained chilled nasal tissues removed from patients undergoing elective ethmoidectomy for chronic rhinosinusitis within 2 hr of removal. Tissues selected for use were not polypoid, infected, or abnormal by gross inspection. We isolated human nasal epithelial (HNE) cells after typical procedures. After overnight digestion of the tissues at 4°C in 0.1% protease in Ham's F-12 medium containing penicillin (100 U/mL), streptomycin (100 µg/mL), fungizone (2.5 µg/mL), and gentamicin (50 µg/mL), we added 10% fetal calf serum to neutralize the protease and freed the epithelial cells from the tissue by agitation and isolated them by centrifugation. We then seeded the washed cells at a density of ≥ 1.6×10^4 cells/cm^2 onto collagen-coated dishes in Ham's F-12 medium containing insulin (5 µg/mL), hydrocortisone (0.5 µg/mL), epidermal growth factor (0.5 ng/mL), triiodothyronine (6.5 ng/mL), retinoic acid (0.1 ng/mL), transferrin (10 µg/mL; Gibco), gentamicin (50 µg/mL), and amphotericin B (50 ng/mL; Gibco). When they reached confluence, we transferred the nasal epithelial cells to Vitrogen-coated 24-well Falcon filter inserts (0.4 µm pore size) and grew them to confluence with Dulbecco's modified Eagle's medium:LHC basal medium (50:50; Biofluids) containing insulin (5 µg/mL), hydrocortisone (70 ng/mL), epidermal growth factor (25 ng/mL), triiodothyronine (6.5 ng/mL), bovine pituitary extract (10 µg/mL), retinoic acid (0.1 ng/mL), bovine serum albumin (0.5 mg/mL), transferrin (10 µg/mL), penicillin (100 U/mL), streptomycin (100 µg/mL), gentamicin (50 µg/mL), and amphotericin B (50 ng/mL), above (200 µL) and below (400 µL) the cells. When cultures were confluent, we removed amphotericin B and gentamicin from the medium and maintained the cultures for 1–4 days before study, with the apical surfaces free of medium. In some instances, we expanded HNE cell numbers through additional passage(s) before use; we used no cells beyond passage 3. To confirm the epithelial cell composition of the primary cultures, we stained cells using a monoclonal mouse antibody against human epithelial-specific antigen (NCL-ESA; Vector Laboratories, Burlingame, CA) and mouse immunoglobulin G_1 (IgG_1) as control. We visualized the presence of antigen on the cell surfaces using a Vectastain ABC kit (Vector Laboratories) with diaminobenzidine tetrahydrochloride (DAB) as a substrate followed by counterstaining with hematoxylin. Similarly stained cells of the WI-38 fibroblast line served as negative controls.

Rhinovirus. We obtained RV16, expanded by infection of WI-38 cells (ATCC), from David Proud at Johns Hopkins Asthma and Allergy Center. This virus stock was previously shown to induce cytokine production by upper and lower respiratory epithelial cells through mechanisms that were RV16 specific and not associated with factors coisolated with the virus (*20*). We purified the virus stock by centrifugation through sucrose gradients. To determine a relative value for the viral dose of this stock required to produce 50% infection of BEAS-2B cells in culture ($TCID_{50}$) in our hands, we infected BEAS-2B cells grown to confluence in 24-well culture inserts in eight replicates with 100 µL of log dilutions of the stock virus for 1 hr. We then washed cultures free of unbound virus and incubated them at 34°C for 72 hr. We diluted supernatant from each of the freeze-thaw–lysed cultures 1:1 in WI-38 medium and transferred them in duplicate to cultures of WI-38 cells in 96-well plates. After incubation at 34°C for 5 days, we determined the presence of viral infection at each viral dilution by standard cytopathic assay. This determination indicated that 100 µL of the stock viral preparation, when diluted 1:10,000, produced 50% infection of BEAS-2B cells in culture ($TCID_{50}$). We used this stock in all experiments. Unless indicated otherwise, we inoculated cells with a 1:1,000 dilution of the stock preparation (i.e., 10^1 $TCID_{50}$).

Oxidant exposure system. We randomly assigned confluent epithelial cell cultures grown at the air–liquid interface to groups for a single exposure to air (5% CO_2 in air) as control or to the oxidants NO_2 (2.0 ppm) or O_3 (0.2 ppm) for 3 hr. We bled nitrogen dioxide through a metering system into one of two matched 6-L Plexiglas exposure chambers, each of which received a flow of 5% CO_2 in air at a rate of 1 L/min. Before this mixing, we saturated the 5% CO_2 in air with water at 40°C to allow the air entering the chamber to achieve > 97% relative humidity at 37°C, despite the addition of the dry NO_2. We monitored the concentration of NO_2 in the chamber continuously with an Interscan monitor and manually adjusted the gas mixture to achieve and maintain the desired concentration. We generated ozone by directing 5% CO_2 in air through a specially fabricated O_3 generator consisting of a glass and stainless steel cylinder containing ultraviolet mercury vapor lamps. An O_3 ultraviolet photometer (model 1008AH; Dasibi, Glendale, CA), modified by Dasibi to operate at a 200 mL/min flow rate, provided continuous measurement of O_3 concentration within the chamber. We sent these values to a computer that used an algorithm to monitor changes in O_3 concentration and to operate a servo-controlled mechanism that regulated the proportion of the total airflow to the chamber that first passed through the generator. This system rapidly established and consistently maintained the concentration of O_3 at the desired level throughout the exposure period. We maintained the relative humidity of the inflow air and air within the matched exposure chambers at > 97% and the temperature at 37°C.

Infection/exposure protocol. We inoculated confluent cultures of BEAS-2B or HNE cells, acclimated to an air interface at their apical surfaces, with RV16 diluted in Hank's balanced salt solution (HBSS). We pipetted the inoculum or HBSS without virus (control) onto the apical surface of each culture. After 1 hr at 34°C, we removed the apical solution and removed the unbound virus by gentle washing with HBSS. We then transferred the cells to the exposure chambers to undergo oxidant or control air exposure for up to 3 hr at 37°C. At the end of exposure, we added medium to the apical surfaces of the cells and returned the cultures to the 34°C incubator, where they remained while we took the required samples of medium and/or cells, as described for the individual experiments.

Preliminary dose–response experiments for NO_2 and O_3 indicated that 3-hr exposures to concentrations from 1.0 to 3.0 ppm and 0.1 to 0.3 ppm, respectively, induced cytokine release from bronchial epithelial cells in a dose-dependent manner with minimal effect on cell viability. For the present study, we used mid-range concentrations of both oxidant gases to facilitate the detection of additive or synergistic interactions with virus infection.

Measurements of IL-8 and ICAM-1. We pooled equal volumes of medium from above and below each culture for assay of IL-8 protein. We determined the concentration of human IL-8 in culture medium using a commercially available ELISA kit (Biosource, Camarillo, CA). The reported level of sensitivity for this assay is < 10 pg/mL, with a linear range of detection between 15.6 and 1,000 pg/mL. All samples fell, or we diluted them to fall, within this range. The antibody is reported by the manufacturer (Biosource) not to cross-react with IL-1β, IL-2, IL-3, IL-4, IL-7, IL-10, IL-13, IFN_γ, TNF_α, or TNF_β. Because neither exposure to the oxidants nor infection with virus at the concentrations employed affected cell number or viability, we report IL-8 release as picograms per milliliter medium. We determined the expression of ICAM-1 on the surfaces of cells in culture using a double-antibody colorimetric detection assay developed in our

laboratory. We washed intact, confluent cultures free of medium with phosphate-buffered saline (PBS) and fixed them with 2% paraformaldehyde in PBS for 10 min at room temperature (RT). We again washed the fixed cultures and incubated them with 5% normal goat serum (NGS; Kirkegaard and Perry Labs, Gaithersburg, MD) in PBS for 1 hr at 37°C. After washing, we incubated cultures with mouse anti-CD54 antibody (1 µg/mL; Immunotech, Westbrook, ME) for 45 min at RT or overnight at 4°C. After washing the cultures with 0.05% Tween 20 in PBS, we incubated them with goat anti-mouse IgG–horseradish peroxidase (1:5,000; American Qualex, San Clemente, CA) in 5% NGS in PBS for 45 min at RT. After a final wash with 0.05% Tween 20 in PBS, we added 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Kirkegaard and Perry) and allowed the color to develop. We stopped the reaction at 20 min with 0.18 M sulfuric acid and read the absorbance at 450 nm. We report ICAM-1 expression as the percentage increase in expression in treated cells above that in air-exposed, noninfected control cultures.

Statistical analyses. We present data as means ± SE. We tested differences between means for group data for significance by the nonpaired Student's *t*-test, and used one-way repeated-measures analysis of variance for comparing cytokine release kinetics. We performed statistical analyses with SigmaStat Statistical software (Jandel Scientific, San Rafael, CA), and considered *p*-values < 0.05 significant.

Results

Characteristics of IL-8 release from BEAS-2B cells. Figure 1A shows the effect of RV16 infection and NO_2 exposure (2.0 ppm), alone or in combination, on the release of IL-8, in excess of that released from control cells. The combined treatment induced marked enhancement of cytokine release above the levels predicted from the modest stimulating effects of the individual treatments. Increases above the predicted additive effects were 42%, 191%, and 250% at the 1:3,000, 1:1,000, and 1:300 dilutions of RV, respectively. In response to control conditions (sham inoculation and air exposure), 37 ± 4 pg/mL of IL-8 was released into the medium. Exposure to O_3 (0.2 ppm), a more highly reactive oxidant, produced a similar enhancement of the RV16-induced release of IL-8 (Figure 1B). This effect was less pronounced than that seen with NO_2, however, resulting in 67%, 44%, and 41% increases above the additive effects at the three dilutions of virus, respectively. Release of IL-8 from cells exposed to control conditions was 65 ± 5 pg/mL.

The release of IL-8 from the cells occurred rapidly in response to each of the stimuli and followed a similar time course. Figure 2 compares the patterns of release in response to virus and NO_2, singly and in combination. We achieved maximal release under conditions of individual stimulation within 6 hr from the end of the 3-hr exposure period (i.e., 9 hr from the end of the 1-hr inoculation period). This time course was not altered by combined stimulation of the cells by virus and oxidant. When expressed as IL-8 released in response to each experimental condition in excess of that under the control condition, the release at the 6-hr and 12-hr time points in response to combined infection and NO_2 exposure was significantly higher than the sum of release from the cells that underwent only infection or only NO_2 exposure. These data confirm, at earlier time points, the synergism between RV16 and NO_2 observed at 18 hr postexposure in separate experiments and depicted in Figure 1A. Further, they demonstrate the consistency of the synergistic enhancement of RV16-induced inflammation by oxidant stimuli.

Responses of HNE cells. HNE cells represent the principal upper respiratory cell in which interactions between RV16 and oxidant pollutants would occur under normal conditions *in vivo*. To confirm that the responses observed in cells of the BEAS-2B line reflected those of primary cells, we examined the release of IL-8 from HNE cells in response to RV16 infection in the presence and absence of subsequent exposure to NO_2 or O_3. Table 1 summarizes these data. Release of the cytokine from control cells during the 18 hr posttreatment period was approximately 3,000 times higher in primary cells compared with BEAS-2B cells (~15 ng/mL vs. ~50 pg/mL, respectively). Inoculation of the cultures with a minimally infective concentration of RV16 (i.e., 10^1 $TCID_{50}$) produced small increases in IL-8 release in the primary cells that, on average, did not reach statistical significance. Nevertheless, when combined with NO_2 exposure (2.0 ppm), the low level of this infective stimulus significantly enhanced the response. The level of release of IL-8 by HNE cells after combined RV16 and NO_2 exposure was significantly greater than the sum of the individual responses (Table 1). Similarly, the combination of RV16 infection and exposure

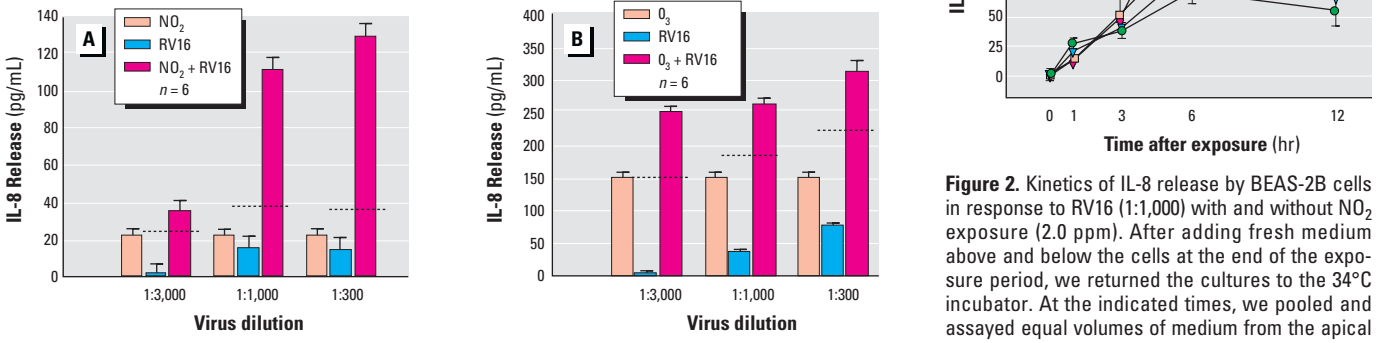

Figure 1. Release of IL-8 by BEAS-2B cells into the medium, in excess of that released by air-exposed and sham-inoculated cells, during an 18-hr period after the end of the exposure period. We added fresh medium above and below the cells at the end of the exposure period and returned the cultures to the 34°C incubator. We pooled and assayed equal volumes of medium from the apical and basal chambers. (*A*) Cultures exposed to air or NO_2 (2.0 ppm, 3 hr); data from two experiments with three cultures per treatment group. (*B*) Cultures exposed to air or O_3 (0.2 ppm, 3 hr); data from four experiments with three cultures per treatment group. Dotted lines indicate the value corresponding to the numerical sum of the individual oxidant and RV16 responses. Values represent concentration mean ± SE for each condition above that released from sham-infected, air-exposed (control) cultures; *n* indicates the number of cultures for each treatment group.

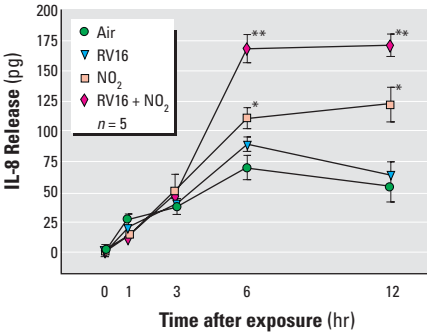

Figure 2. Kinetics of IL-8 release by BEAS-2B cells in response to RV16 (1:1,000) with and without NO_2 exposure (2.0 ppm). After adding fresh medium above and below the cells at the end of the exposure period, we returned the cultures to the 34°C incubator. At the indicated times, we pooled and assayed equal volumes of medium from the apical and basal chambers. Values represent the amount of protein (mean ± SE) cumulatively released into the medium up to each time point; *n* indicates the total number of cultures for each condition from two separate experiments.

*$p < 0.05$ by repeated measures ANOVA in comparison with corresponding air value. **Significant difference ($p < 0.05$) by group *t*-test compared with the numerical sum of individual responses to RV16 and NO_2 at each time point after those values are normalized to the sham-infected, air-exposed controls at the corresponding time points.

to O_3 (0.2 ppm) enhanced cytokine release significantly more than the sum of the individual RV16 and O_3 stimuli. These results are consistent with a synergistic, rather than additive, interaction between RV16 and the two oxidant gases, similar to the effect demonstrated in the BEAS-2B cells (Figure 1).

To determine whether a positive interaction between RV16 and NO_2 activates other proinflammatory cell proteins, we also determined expression of ICAM-1 on the apical surfaces of infected and/or exposed HNE cells. We grew and treated primary cultures according to a protocol identical to that used for the IL-8 experiments, and assessed ICAM-1 expression 18 hr after the exposure period. Table 2 summarizes the results of two experiments. We observed substantial expression of cell surface ICAM-1 by unstimulated HNE cells in culture. Because cell preparations varied in their responses to stimulation by virus or NO_2, the data from the experiments are more meaningfully presented as percentage increases above control cells for each set of cultures. However, the pattern of increased ICAM-1 expression shown in Table 2 mirrors that seen in preliminary experiments in which we infected cultures or exposed them to NO_2 independently. Baseline expression was increased to a statistically significant extent ($p < 0.05$) by both virus and oxidant. The effects of RV16 and NO_2 combined to enhance cell surface expression to an extent that appeared on average to be additive in primary cultures of HNE cells.

Inhibition of oxidant-dependent pathways. To confirm the involvement of oxidant-mediated stimulation in the synthesis of IL-8 in response to treatments of BEAS-2B cells with RV16, NO_2, and O_3, we treated cultures with the antioxidant and reactive oxidant scavenger *N*-acetylcysteine (NAC; Sigma) in a range of concentrations for 30 min before inoculation and throughout the subsequent exposure and 18-hr postexposure incubation period. NAC inhibited cytokine release in response to NO_2 (1.0 ppm) and O_3 (0.1 ppm) exposures in a similar and dose-dependent manner (Figure 3). RV16-induced IL-8 release was also inhibited in a dose-dependent manner by the antioxidant, but to a lesser extent than were the two oxidant gases. At a concentration of NAC that produced maximal inhibition of the effects of oxidant exposures (40 mM), the combine effects of RV16 with each of the oxidants at the higher concentrations used in the previous experiments were reduced to levels equivalent to or below those observed in nontreated, unexposed cells (Figure 3B). Preliminary experiments indicated that, when we adjusted treatment solutions to a pH of 7.4, this relatively high concentration of NAC had no effect on cell viability as determined by trypan blue dye exclusion (control, 93.8 ± 1.6%; NAC, 95.2 ± 0.5%). It is possible that, in addition to its actions as an intracellular antioxidant, NAC treatment directly reduced the interaction of NO_2 and O_3 with the cells or interfered with viral infectivity through undetermined mechanisms. Nevertheless, these data provide compelling evidence for the importance of oxidant-related pathways in the release of the cytokine by each of the stimuli. Under conditions of combined infection/exposure and of oxidant exposure alone, reduction of oxidant stress within the cells by NAC treatment inhibited IL-8 release to statistically equivalent levels (NO_2 + RV16 + NAC vs. NO_2 + NAC, 46.9 ± 5.4 pg/mL vs. 42.1 ± 4.6 pg/mL; O_3 + RV16 + NAC vs. O_3 + NAC, 20.8 ± 2.4 pg/mL vs. 19.3 ± 4.0 pg/mL; all $p > 0.05$; not shown in Figure 3).

Discussion

As evidence more strongly supports the direct action of rhinovirus in the lower airways in triggering asthma exacerbations (*21*), understanding the influence of environmental agents on this process that also target the peripheral airways becomes increasingly important. The present study demonstrates that the activation of proinflammatory pathways within cells of both the upper and lower airway epithelium induced by infection with RV16 can be significantly enhanced when the cells are also exposed to the common environmental oxidant pollutants NO_2 and O_3. We found the enhanced expression of the neutrophil chemotactic factor IL-8 to be more than additive, indicating a synergistic interaction of the stimuli leading to cytokine production. We also observed an interactive effect of these stimuli on expression of ICAM-1, a protein involved in a wide range of inflammation-associated binding processes at the epithelial surface, at a level that was at least additive.

Interactions between distinct chemical and/or biologic agents that enhance the expression of mediators of the inflammatory process are not unusual. Steady-state levels of IL-8 mRNA in monocytes treated with lipopolysaccharide (LPS) were shown to be synergistically increased by preconditioning the cells with oxidant stress (*22*), and expression of ICAM-1 on human bronchial epithelial cells was more than additively increased by combined stimulation with TNF_α and IFN_γ (*23*). In the case of several cytokines, especially IL-8, increased gene expression has been associated with the activity of at least two transcription factors working cooperatively to transactivate the gene, for example, nuclear factor (NF)-κB and NF-IL-6, CCAAT/enhancer binding protein (C/EBP), or activator protein (AP)-1 (*16,19,24–26*). Thus, the diversity of signaling and activation pathways within most cells provides ample opportunity for significantly amplifying the inflammatory response in the presence of multiple stimuli.

In the present study, we saw synergistic interaction between RV16 infection and chemical oxidant exposure, two naturally occurring environmental challenges to the airway epithelium. The pathway shared by these two stimuli likely involves the development of oxidative stress within the epithelial cells and the activation of oxidant-responsive transcription factors. In our studies, treatment of the cells with the antioxidant NAC markedly reduced production of IL-8 in

Table 1. Cumulative release of IL-8 (ng/mL) from primary cultures of HNE cells in response to RV16 infection in the presence or absence of NO_2 or O_3 exposure during 18 hr after the exposure period.

	RV16	Oxidant	RV16 + oxidant
NO_2	1.77 ± 0.75	6.21 ± 0.81*	13.00 ± 0.67 **
O_3	1.13 ± 0.61	8.34 ± 0.64*	14.16 ± 0.75 **

NO_2 data are from three separate experiments, each using cells from different tissue sources and containing five to six cultures per treatment group; O_3 data are from two separate experiments, each using cells from different tissue sources and each containing five to six cultures per treatment group. Immediately following the 3-hr oxidant exposure period, we added fresh medium to the culture inserts above and below the cells and returned the cultures to the 34°C incubator. Then, after 18 hrs we pooled the medium from above and below the cells for assay of IL-8. Values represent mean ± SE of cumulative release in excess of that seen in sham-infected and air-exposed (control) cultures. Control release: NO_2, 18.59 ± 1.56 ng/mL; O_3, 12.37 ± 1.13 ng/mL.
*Significant ($p < 0.05$) increase in release above control values by group *t*-test. **Significant ($p < 0.001$) increase in release compared with the numerical sum of individual RV16 and oxidant treatments.

Table 2. Increased expression of ICAM-1 (%) on the apical surfaces of primary HNE cells in response to RV16 infection and/or NO_2 exposure 18 hr after the exposure period.

Tissue source	RV16	NO_2	RV16 + NO_2
1	9.5 ± 1.5*	14.4 ± 2.1*	31.1 ± 2.4*
2	13.5 ± 6.9	31.5 ± 4.7*	43.4 ± 7.1*

Data are from two separate experiments. Values represent individual mean ± SE of the percentage increase in expression in excess of that seen in sham-infected, air-exposed (control) cultures for each of the experiments. Six cultures per treatment condition and control in each experiment, three from each of two different tissue sources.
*Significant ($p < 0.01$) increase in expression above control values within each experiment.

response to RV16 alone and to NO_2 and O_3 alone or in combination with RV16. Environmental pollutants with oxidant properties, such as NO_2 and O_3, are well known to exert their effects through the generation of oxidant stress within airway cells (*27*), and reactive oxidant species are known to play an especially central role in the regulation of IL-8 production in these cells (*28*). Furthermore, transcription factors such as NF-κB, AP-1, and NF-IL-6 (*19,24,29*) are known to mediate oxidant-related stimuli leading to synthesis of IL-8, ICAM-1, and other proinflammatory mediators (*16,29*). In a study that assessed the effects of O_3 exposure on IL-8 expression in cells of the A549 type II line, electrophoretic mobility shift assay indicated that binding of all three of these factors, NF-κB, AP-1, and NF-IL-6, to the IL-8 promoter accompanied increases in IL-8 mRNA and protein levels (*30*).

The mechanisms through which RV16 infection stimulates IL-8 expression are less clear. We know that attachment of members of the major receptor group of rhinoviruses, such as RV16, to their ICAM-1 receptors on the epithelial cell surface is required for increased IL-8 production (e.g., *13,31*). Furthermore, we have evidence that as much as half of this stimulatory effect may be related to the binding of the virus to the cell surface (*32*). Our NAC data suggest that a significant portion of the effect on cytokine release, whether related to binding or to events involved with viral replication, is associated with the generation of reactive oxidant species within the cells.

Consistent with the results of *in vivo* studies in our laboratory (*33*) and many others, we observed significant upregulation of ICAM-1 in epithelial cells exposed to NO_2 or O_3. Increased cell surface expression of ICAM-1 caused by RV16 infection has also been reported previously (*34,35*), and in one of two experiments we observed this in cells that underwent RV16 infection alone. The use of minimally infective concentrations of RV16 in our studies likely accounts for the inconsistency of these ICAM-1 data and is also reflected in the lower levels of IL-8 released by the cells in our studies compared with results of others. To increase the range for potential synergistic interaction between RV16 and the oxidants, we used concentrations of virus (10^1 $TCID_{50}$ per culture) that are 1,000 times lower than those that have been used to initiate maximal release of IL-8 in human airway epithelial cells (*36*). Nevertheless, the time course of cytokine release reported in the present studies is similar to that observed with the higher virus titers (*36*).

Although our data indicate that mechanisms involving generation of reactive oxidant species are likely to play a significant role in the synergy of action between RV16 and NO_2 or O_3, the possibility exists that some contribution could also come from activation of a parallel nonoxidant pathway. Such appears to be the case in the stimulation of rat lung epithelial cells by TNF_α and H_2O_2 (*37*). In that study, in which these two stimuli were shown to cooperate to activate NF-κB, two separate pathways consisting of different downstream effectors were indicated to be involved. The incomplete inhibition of NO_2-induced IL-8 release by NAC in the present study suggests that the effects of this pollutant may not be mediated solely through oxidant-associated pathways.

We currently know little about the specific mechanisms through which NO_2 stimulates cytokine release; however, the difference in oxidative capacity between NO_2 and the much stronger O_3 could provide the basis for the activation of different response elements by the two oxidants.

In summary, the present study demonstrates that exposure of epithelial cells of the upper and lower respiratory tract to the environmental oxidant pollutants NO_2 and O_3 markedly enhances their RV16-induced expression of proinflammatory activity. Epithelium in both of these regions is a recognized common target of action for these frequently occurring exposures under normal conditions. Studies in children and adults with asthma have shown RV16 to be the most common viral precipitant of acute attacks (*5,6*), and that lower airway inflammatory responses to the virus may play a causal role in the triggering process (*10*). In addition, earlier studies suggesting that rhinovirus infection increases the level of subsequent allergen-induced inflammation (*38*) continue to find support in the results of more recent investigations. However, we do not presently know the extent to which indoor and outdoor oxidant pollutants may enhance these effects of rhinovirus in allergic asthmatics. Carefully designed investigations involving this subpopulation will be necessary to determine if the interaction between oxidant pollutants and rhinovirus infection described in the present studies provides a mechanism through which environmental exposures may pose an important health risk in these individuals.

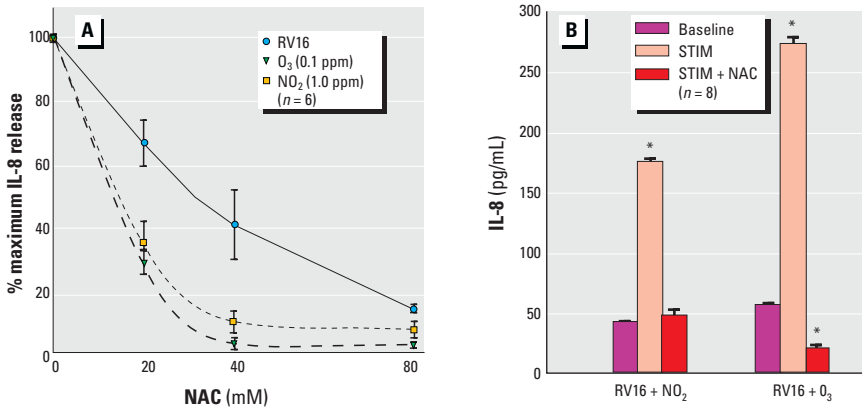

Figure 3. Dose-dependent inhibition of IL-8 release in response to (*A*) RV16, O_3, or NO_2 by treatment of BEAS-2B cells with NAC, an antioxidant and free radical scavenger. Twelve groups of six cultures each underwent sham or active treatment for 30 min before the inoculation or exposure procedures and throughout the exposures and 18-hr postexposure period. (*B*) In a separate experiment using the lowest concentration causing maximal inhibition of O_3 and NO_2 (40 mM), IL-8 release in response to the combined effects of RV16 with NO_2 and of RV16 with O_3, at exposure concentrations of 2.0 ppm and 0.2 ppm, respectively, were reduced to levels at or below spontaneous release (baseline) over the same time period. Values represent the amount of protein (mean ± SE) released into the medium during the 18-hr postincubation period; *n* indicates the number of cultures in each of the six treatment groups.
*$p < 0.001$ by group *t*-test compared with corresponding baseline value.

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