

## Diesel Exhaust Enhances Influenza Virus Infections in Respiratory Epithelial Cells

Iлона Jaspers,<sup>\*,†,‡,1</sup> Jonathan M. Ciencewicki,<sup>†</sup> Wenli Zhang,<sup>\*</sup> Luisa E. Brighton,<sup>\*</sup> Johnny L. Carson,<sup>\*,‡</sup> Melinda A. Beck,<sup>‡</sup> and Michael C. Madden<sup>†,§</sup>

<sup>\*</sup>Center for Environmental Medicine, Asthma, and Lung Biology; <sup>†</sup>Curriculum of Toxicology; <sup>‡</sup>Department of Pediatrics, Division of Infectious Diseases and Host Defense; University of North Carolina at Chapel Hill, North Carolina 27599–7310; and <sup>§</sup>U.S. Environmental Protection Agency, Human Studies Division; Chapel Hill, North Carolina 27599

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Several factors, such as age and nutritional status, can affect the susceptibility to influenza infections. Moreover, exposure to air pollutants, such as diesel exhaust (DE), has been shown to affect respiratory virus infections in rodent models. Influenza virus primarily infects and replicates in respiratory epithelial cells, which are also a major targets for inhaled DE. Using *in vitro* models of human respiratory epithelial cells, we determined the effects of an aqueous-trapped solution of DE (DE<sub>as</sub>) on influenza infections. Differentiated human nasal and bronchial epithelial cells, as well as A549 cells, were exposed to DE<sub>as</sub> and infected with influenza A/Bangkok/1/79. DE<sub>as</sub> enhanced the susceptibility to influenza virus infection in all cell models and increased the number of influenza-infected cells within 24 h post-infection. This was not caused by suppressing antiviral mediator production, since interferon (IFN)  $\beta$  levels, IFN-dependent signaling, and IFN-stimulated gene expression were also enhanced by exposure to DE<sub>as</sub>. Many of the adverse effects induced by DE exposure are mediated by oxidative stress. Exposure to DE<sub>as</sub> used in these studies generated oxidative stress in respiratory epithelial cells, and addition of the antioxidant glutathione-ethylester (GSH-ET) reversed the effects of DE<sub>as</sub> on influenza infections. Furthermore, DE<sub>as</sub> increased influenza virus attachment to respiratory epithelial cells within 2 h post-infection. Taken together, the results presented here suggest that in human respiratory epithelial cells oxidative stress generated by DE<sub>as</sub> increases the susceptibility to influenza infection and that exposure to DE<sub>as</sub> increases the ability of the virus to attach to and enter respiratory epithelial cells.

**Key Words:** influenza; diesel exhaust; *in vitro*; epithelial cells; oxidative stress.

Although many sources contribute to the overall ambient particulate matter (PM) levels, especially in urban environ-

Human Subjects: The protocols for the acquisition of both primary human bronchial and nasal epithelial cells were reviewed by the University of North Carolina Institutional Review Board and informed written consent was obtained from all subjects.

<sup>1</sup>To whom correspondence should be addressed at CEMALB, CB#7310, 104 Mason Farm Rd., University of North Carolina at Chapel Hill, Chapel Hill, NC 27599–7310. Fax: (919) 966-9863. E-mail: Iлона\_Jaspers@med.unc.edu.

ments, diesel exhaust (DE) can be a significant factor in PM levels. DE particles (DEP) consist of a carbonaceous core with many different adsorbed organic materials, especially polycyclic aromatic hydrocarbons (PAH), which are thought to be responsible for eliciting many of the adverse health effects associated with exposure to DE. For example, Hiura *et al.* (1999, 2000) have demonstrated that both DEP and organic extracts of DEP are capable of generating reactive oxygen species and apoptosis in macrophages. Furthermore, it has been suggested that PAH adsorbed onto the particle surface are responsible for the pro-inflammatory and tissue damaging effects of DEP, which can be mitigated by the addition of antioxidants (Takizawa *et al.*, 2000, 2003). Specifically, stimulation of bronchial epithelial cells with suspended DEP increased the expression of the pro-inflammatory chemokine IL-8, which was mediated by the transcription factor NF- $\kappa$ B and which was attenuated by the addition of the antioxidants N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (Hashimoto *et al.*, 2000; Takizawa *et al.*, 2000). Taken together, these and several other studies suggest that generation of oxidative stress is an important component of the adverse health effects induced by exposure to DE.

Numerous studies have demonstrated that exposure to DE has adjuvant activity and enhances allergic sensitization in laboratory animals and human volunteers (Pandya *et al.*, 2002; reviewed by D'Amato, 2002), by increasing the levels of allergen-specific IgE and pro-allergic cytokines (IL-4, IL-5, IL-6, IL-10, and IL-13) (Diaz-Sanchez, 1997). Hence, DE has important effects on adaptive immune responses. Furthermore, several studies have demonstrated that exposure to DE decreases bacterial clearance and modifies pulmonary macrophage function (Castranova *et al.*, 2001; Saito *et al.*, 2002a; Steerenberg *et al.*, 2004; Yang *et al.*, 2001; Yin *et al.*, 2002), both potentially contributing to decreased host defense and increased susceptibility to microbial infections. Interestingly, fewer studies have examined the effects of DE on viral infections. Hahon *et al.* (1985) have shown that mice chronically exposed to DE for 6 months and infected with influenza virus

have decreased ability to produce interferon (by 78%) and depressed viral killing, which led to increased viral multiplication. Recently, Harrod *et al.* (2003) demonstrated that exposure of mice to diesel engine emissions for 7 days enhanced the pro-inflammatory response to a subsequent infection with respiratory syncytial virus (RSV) and increased the expression of RSV genes without decreasing interferon levels. This study also showed that exposure to diesel engine emission decreased the expression of innate immune defense mediators, specifically surfactant protein A (SP-A) and Clara Cell Secretory Protein (CCSP), which are important in controlling RSV infections (LeVine *et al.*, 1999; Wang *et al.*, 2003). Taken together, these studies indicate that both chronic and subchronic exposures to DE can enhance the susceptibility to viral infections, possibly through two different mechanisms.

Despite large-scale vaccination efforts and antiviral therapies, morbidity and mortality associated with influenza infections have not significantly changed over the past several years (Thompson *et al.*, 2003, 2004). Factors such as age, nutritional status, and preexisting pulmonary disease can affect susceptibility to influenza (Beck and Matthews, 2000). In addition, the studies by Hahon *et al.* (1985) and Harrod *et al.* (2003) suggest that exposure to DE can also affect the susceptibility to viral infections. Airway epithelial cells are the primary site for influenza virus infection and replication. Virus-infected epithelial cells respond to influenza infection by synthesizing and releasing numerous cytokines and immunoregulatory mediators, which recruit and activate inflammatory cells to aid in the defense and the clearance of the invading virus. Among the mediators released by epithelial cells upon influenza infection, RANTES, MCP-1, IL-8, IL-6, and eotaxin recruit and activate pro-inflammatory cells, while type I interferons (IFN $\alpha$  and IFN $\beta$ ) induce the synthesis and activity of mediators involved in turning off viral replication within the host cell. Both groups of mediators are essential in the successful clearance of an influenza infection. However, other host cell-derived factors also control the susceptibility to viral infections. For example, innate immune defense mediators, such as calcium-dependent collagen-like lectins (collectins) released by epithelial cells, can aggregate the viral pathogen, thus neutralizing the ability of the virus to attach and infect the host cell.

Differentiated primary human respiratory epithelial cells, when grown under defined culture condition (see Methods), retain many characteristics seen *in vivo* (Clark *et al.*, 1995; Gray *et al.*, 1996; Ostrowski *et al.*, 1995), including the presence of ciliated, nonciliated, and mucus-secreting cells, as well as tight junctions (Clark *et al.*, 1995) and the ability to produce innate immune defense mediators (Hawgood *et al.*, 2004; Wu *et al.*, 1986). This study was designed to examine whether acute exposures to an aqueous-trapped solution of DE (DE<sub>as</sub>) can affect influenza infections in human respiratory epithelial cells, using *in vitro* models of differentiated human nasal and bronchial epithelial cells as well as a respiratory

epithelial cell line. Our data demonstrate that acute exposure to DE<sub>as</sub> increases the number of influenza-infected cells in an oxidative stress-dependent manner, and that this may be caused by the ability of DE<sub>as</sub> to increase influenza virus attachment and possibly entry into epithelial cells.

## MATERIALS AND METHODS

**Cell culture.** A549 cells, a human pulmonary type II epithelial-like cell line, were cultured in F12K medium plus 10% fetal bovine serum and 1% penicillin and streptomycin (all from Invitrogen, Carlsbad, CA). For treatment with DE<sub>as</sub> and infection with influenza, A549 cells were grown in 6- or 12-well plates. When the cells reached about 70–80% confluency and about 18–24 h before exposure to DE<sub>as</sub> and infection with influenza, the cell culture media was exchanged for serum-free F12K plus 1.5  $\mu$ g/ml BSA plus antibiotics. In some experiments, a cell-permeable form of reduced glutathione (glutathione-ethyl ester; GSH-ET; 10mM; Sigma, St. Louis, MO) was added 30 min before exposure to DE<sub>as</sub>.

Primary human bronchial cells were obtained from healthy nonsmoking adult volunteers by cytologic brushing at bronchoscopy. Primary human nasal cells were obtained from healthy nonsmoking adult volunteers by gently stroking the inferior surface of the turbinate several times with a Rhino-Probe curette (Arlington Scientific, Arlington, TX), which was inserted through an otoscope with a large aperture. The protocols for the acquisition of both primary human bronchial and nasal epithelial cells were reviewed by the University of North Carolina Institutional Review Board and informed written consent was obtained from all subjects. Both primary human bronchial and nasal epithelial cells were expanded to passage 2 in bronchial epithelial growth medium (BEGM, Cambrex Bioscience Walkersville, Inc., Walkersville, MD) and then plated on collagen-coated filter supports with a 0.4  $\mu$ m pore size (Trans-CLR; Costar, Cambridge, MA) and cultured in a 1:1 mixture of bronchial epithelial cell basic medium (BEBM) and DMEM-H with Single-Quot supplements (Cambrex), bovine pituitary extracts (13 mg/ml), bovine serum albumin (BSA, 1.5  $\mu$ g/ml), and nystatin (20 units). Upon confluency, all-*trans* retinoic acid was added to the medium, and air liquid interface (ALI) culture conditions (removal of the apical medium) were created to promote differentiation. Mucociliary differentiation was achieved after 18–21 days post-ALI.

**Exposure to aqueous-trapped solution of diesel exhaust (DE<sub>as</sub>).** DE<sub>as</sub> was generated as described before (Madden *et al.*, 2003). Briefly, we used emissions from a Caterpillar diesel engine, model 3304, which was used to power a 113 KW generator. This type of engine was chosen because it is used in nonroad vehicles, which are significant contributors to ambient diesel exhaust levels, and because the projected trend for emissions from nonroad diesel engines is expected to remain at the same level or even increase in the future (U. S. EPA, 2002). The diesel exhaust emissions from this Caterpillar diesel engine were passed through a tubing system with a filter impactor and two impinger tubes (containing 100 ml PBS each) submerged in an ice bath. Impinger glassware was washed and heated to remove and destroy endotoxin. Of the two impinger tubes, the emissions (at 10 l/min) that entered and remained in the first (primary) tube, but not the secondary tube, were utilized for the cell exposure studies. Extracts were generated and collected during a one-hour period when the engine was under high load (HL). This type of preparation was chosen because it contains DE particles, as well as polar and thus water soluble DE gas-phase components. To determine the mass of the emissions retained within the PBS in an impinger tube, an aliquot was dried overnight at 56°C and corrected for the mass of the PBS contribution (which was determined in a similar manner by overnight drying) and dilution with water from the exhaust. Aliquots of the DE<sub>as</sub> were kept at –20°C until use.

For all cell types used in this study, DE<sub>as</sub> was added 2 h before infection with influenza. Specifically, for the differentiated human nasal and bronchial

epithelial cells, DE<sub>as</sub> was diluted in 200 µl media to achieve 22 or 44 µg DE<sub>as</sub> per cm<sup>2</sup> of cell layer and added to the apical side. After the 2-h incubation with DE<sub>as</sub>, the diluted DE<sub>as</sub> was removed, and influenza virus diluted in the same volume of media was added to the apical side for 2 h, after which it was removed to establish ALI culture conditions again. For the experiments using A549 cells, DE<sub>as</sub> was diluted in F12K media plus BSA plus antibiotics to achieve 6.25, 12.5, or 25 µg/cm<sup>2</sup> and added to the cells. After 2-h incubation with DE<sub>as</sub>, influenza virus was added to the cells. The effects of exposure to DE<sub>as</sub> on cell viability were assessed by analyzing cell culture supernatants for lactate dehydrogenase (LDH) activity using a commercially available kit according to the supplier's instructions (CytoTox 96®, Promega, Madison, WI).

**Infection with influenza.** Throughout this study we used influenza A/Bangkok/1/79 (H3N2 serotype) which was propagated in 10-day-old embryonated hen's eggs. The virus was collected in the allantoic fluid and titered by 50% tissue culture infectious dose in Madin-Darby canine kidney cells and hemagglutination as described before (Beck *et al.*, 2001). Stock virus was aliquoted and stored at -80°C until use. Unless otherwise indicated, for infection of differentiated bronchial or nasal cells as well as A549 cells, approximately 3 × 10<sup>5</sup> cells were infected with 320 hemagglutination units (HAU) of influenza A Bangkok 1/79.

**RT-PCR.** Total RNA was extracted using TRizol (Invitrogen) as per the supplier's instruction. First-strand cDNA synthesis and real-time RT-PCR was performed as described previously (Jaspers *et al.*, 1999, 2001). The sequences for the primers and probes used in this study are as following:

Hemagglutinin (HA): probe, 5'-FAM-TGATGGGAAAACTGCACAC-TGATAGATGC-TAMRA-3'; sense, 5'-CGACAGTCTCACCGAATCC-3'; antisense, 5'-TCACAATGAGGGTCTCCCAATAG-3'; IFNβ: probe, 5'-FAM-AGCAGCAATTTTCAGTGTCCAGAGCTCTG-TAMRA-3'; sense, 5'-CAACTTGCTTGGATTCCTACAAAG-3'; antisense, 5'-AGCCTCCATTCAATTGCC-3'; MxA: probe, 5'-FAM-AGGCCAGCAAGCGCATCTCCAG-TAMRA-3'; sense, 5'-CAGCACCTGATGGCCTATCAC-3'; antisense, 5'-CATGAAGAACTGGATGATCAAAGG-3'; GAPDH: probe, 5'-JOE-CAAGCTTCCCGTTCTCAGCC-TAMRA-3'; sense, 5'-GAAGGTGAAGTCCGGAGTC-3'; antisense, 5'-GAAGATGGTATGGGATTC-3'.

**Virus attachment assay.** A549 or differentiated nasal epithelial cells were exposed to DE<sub>as</sub> and infected with influenza as described above. Nonattached virus was removed by rinsing the cells twice with media either immediately (t = 0) or at 15, 30, 60, or 120 min post-infection. RNA from the rinse containing the nonattached virus was isolated using a Viral RNA Isolation kit (QIAamp Viral RNA Isolation Kit, Qiagen, Valencia, CA) and analyzed for HA RNA levels using gene-specific primer anchored RT-PCR (QuantiTect™ Probe RT-PCR Kit, Qiagen). HA RNA levels at the specific time points were normalized to the HA RNA levels at t = 0 of the respective exposure group. Decreased HA RNA levels would indicate less nonattached virus and are used here as a measure of increased virus attachment.

**Western blotting.** Whole cell lysates were prepared by lysing the cells in RIPA buffer containing 1% Nonidet P (NP)-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors (Cocktail Set III; Calbiochem, San Diego, CA). Nuclear extracts were prepared as described before (Jaspers *et al.*, 2001). Fifty micrograms of whole cell lysate or 20 µg of nuclear extract was separated by SDS-PAGE as described before (Jaspers *et al.*, 2001). This was followed by immunoblotting using specific antibodies to tyr-701 phospho-specific STAT1 (1:1000, Cell Signaling, Beverly, MA), interferon-stimulated gene factor 3 gamma (ISGF3γ; 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), or influenza A H3N2 (1:500; US Biologicals, Swampscott, MA). Antigen-antibody complexes were stained with anti-rabbit horseradish peroxidase-conjugated antibody (1:2000, Santa Cruz Biotechnology) and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The chemiluminescent signals were acquired using a 16-bit CCD camera (GeneGnome system; Syngene, Frederick, MD) and analyzed using the GeneSnap software (Syngene).

**Promoter-reporter assays.** An interferon-stimulated response element (ISRE)-dependent promoter reporter construct (pISRE-luc; Stratagene, La

Jolla, CA) was used. A constitutively active SV-40 promoter-β-galactosidase construct (β-gal; Promega, Madison, WI) was used to adjust for well-to-well variation in cell number and transfection efficiency. A549 cells grown to about 50–80% confluence in 24-well tissue culture dishes were transfected with 500 ng of the pISRE-luc plasmid using 0.75 µl FuGENE6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany). Twenty-four h post-transfection, the cells were serum-starved for 24 h and exposed to DE<sub>as</sub> and infected with influenza as described above. Luciferase and β-galactosidase activities were determined using the Dual Light Reporter assay System (Perkin Elmer) and an AutoLumat LB953 luminometer (Berthold Analytical Instruments, Nashua, NH). Luciferase activity was normalized to β-galactosidase activity and expressed as fold induction over infected but nonexposed cells.

**Immunohistochemistry.** A549 cells were grown on chamber slides (Lab-Tek® Chamber slides, Nalge Nunc International, Naperville, IL) and exposed to DE<sub>as</sub> and infected with influenza as described above. In some experiments, cells were treated with GSH-ET (10 mM; Sigma) 30 min before exposure to DE<sub>as</sub>. At 24 h post-infection, cells were acetone fixed and stained using FITC-tagged mouse anti-influenza A/Texas antibody (10 µg/ml; ViroStat, Portland, ME), which recognizes nucleoprotein of H3N2 influenza A. Samples were washed twice with phosphate-buffered saline and photographed on a Nikon Microphot-SA fluorescence microscope using standard fluorescein excitation and emission filter sets. To determine the average number of influenza-infected cells per microscopic field, a protocol for a systematic randomization procedure was applied. Briefly, each immunohistochemically stained sample was divided into four quadrants. Influenza-infected cells were counted in one field per quadrant as well as one field in the center of the sample. Every field was checked for confluency using phase contrast microscopy. Only fields in which cells reached >90% confluency were considered appropriate for counting, and fields with less than 90% confluent cell monolayer were excluded from the analysis.

**Analysis of oxidative stress.** Protein carbonyl levels were detected by immunoblotting using the OxyBlot™ Protein Oxidation Kit (Chemicon, Temecula, CA) as per the supplier's instruction. Briefly, cells were lysed in RIPA buffer containing 1% Nonidet P (NP)-40, 0.5% deoxycholate, 0.1% SDS, protease inhibitors (Cocktail Set III; Calbiochem), and 50 mM dithiothreitol (DDT). The carbonyl groups in 5 µl cell lysates were derivatized to 2–4-dinitrophenyl hydrazone (DNP-hydrazone) by reaction with 2–4-dinitrophenyl hydrazine (DNPH). The DNP-derivatized protein samples were separated by 12% SDS-PAGE, followed by immunoblotting with antibodies against the DNP moiety of the proteins. Antigen-antibody complexes were stained and visualized as described above. To assure equal loading, blots were stripped using Blot Restore Membrane Rejuvenation Kit (Chemicon) as per the supplier's instruction, and re-probed using antibodies against α-tubulin (Sigma).

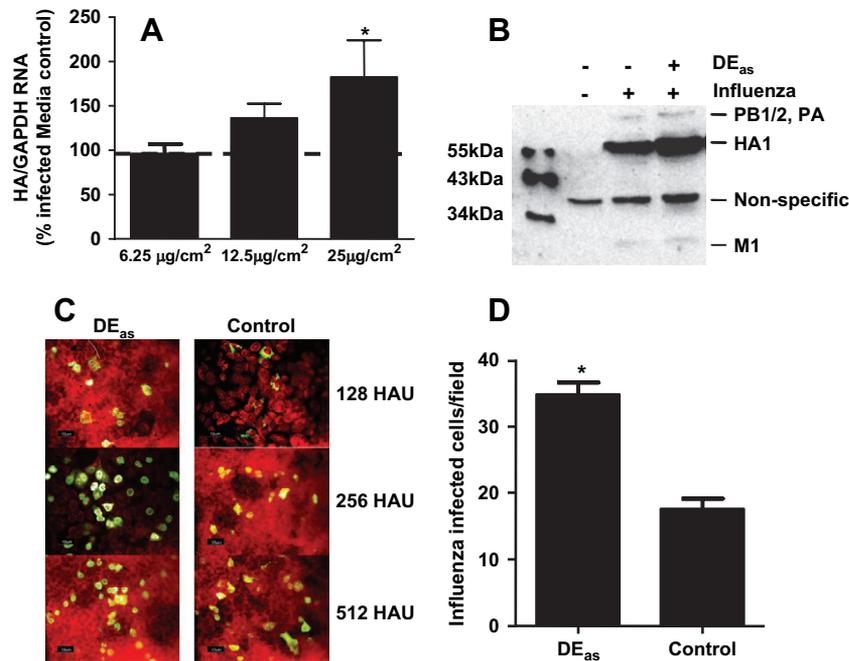
Analysis of oxidative stress using 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) in A549 cells was conducted as described before (Jaspers *et al.*, 2000).

**Statistical Analysis.** Data are expressed as means ± SEM of at least three separate experiments. The RT-PCR and promoter reporter data were expressed as percent of the infected but nonexposed control and analyzed using the Wilcoxon Signed Rank Test, assuming a theoretical mean of 100. All other data were analyzed using a two-tailed Student's *t*-test (Fig. 1D) or one-way analysis of variance followed by the Newman-Keul's *post hoc* test for multigroup analysis. A value of *p* < 0.05 was considered to be significant.

## RESULTS

### Exposure to DE<sub>as</sub> Increases Susceptibility to Influenza Infections in A549 Cells

Following viral entry, the influenza virus uncoats, the viral nucleoprotein core enters the nucleus, and replication and



**FIG. 1.** Exposure to DE<sub>as</sub> enhances susceptibility to influenza infections in A549 cells. A549 cells were exposed to 6.25, 12.5, or 25 µg/cm<sup>2</sup> DE<sub>as</sub> for 2 h and infected with influenza A/Bangkok/1/79. (A) Total RNA was analyzed for viral HA RNA levels using real-time RT-PCR at 24 h post-infection. HA RNA levels were normalized to GAPDH mRNA levels and expressed as percent of the respective influenza-infected media control. (B) Whole cell lysates of A549 cells exposed to 25 µg/cm<sup>2</sup> DE<sub>as</sub> for 2 h and infected with influenza A were analyzed for influenza virus proteins using an influenza A H3N2 serotype antibody at 24 h post-infection. Representative immunoblots are shown. (C) A549 cells were exposed to 25 µg/cm<sup>2</sup> DE<sub>as</sub> for 2 h and infected with influenza A/Bangkok/1/79 at an M.O.I. of 128, 256, and 512 HAU per  $3 \times 10^5$  cells. At 24 h post-infection, cells were acetone-fixed and visualized using a FITC-tagged anti-influenza A antibody. Direct immunofluorescent localization of influenza-infected A549 cells. (D) Average number of immunofluorescent cells counted in six different microscopic fields from A549 cells infected with 256 HAU after exposure to DE<sub>as</sub>. Data are expressed as mean  $\pm$  SEM. \*Significantly different from the influenza-infected nonexposed media control;  $p > 0.05$ .

transcription of viral RNA takes place. Real-time RT-PCR of viral RNA levels has been shown to have similar sensitivity and specificity as compared to conventional methods for detecting influenza virus titers, such as hemagglutination inhibition assays (Spackman *et al.*, 2002). Because of the ability to deal with large number of samples in a rapid and sensitive manner, we used real-time RT-PCR of HA RNA as a measurement of viral replication and level of infection in respiratory epithelial cells. We synthesized primers and probes specific for influenza A/Bangkok/1/79 HA viral RNA and analyzed HA RNA levels in A549 cells. HA RNA levels were normalized to GAPDH mRNA levels and expressed as percent influenza-infected but non-DE<sub>as</sub>-exposed control. Figure 1A shows that treatment with DE<sub>as</sub> enhances HA RNA levels in A549 cells in a dose-dependent manner. Specifically, exposure to 25 µg/cm<sup>2</sup> DE<sub>as</sub> significantly enhanced HA RNA levels in A549 cells. To confirm that enhanced viral RNA levels seen in Figure 1A also resulted in enhanced viral transcription and translation into viral protein levels, we analyzed whole-cell lysates for the appearance of viral proteins. Using a polyclonal antibody to influenza A H3N2, the serotype of influenza A/Bangkok/1/79, we determined whether treatment with DE<sub>as</sub> enhances viral protein levels in A549 cells. Based on the molecular weight, we estimate the proteins detected by this polyclonal antibody to be

the subunits for the viral RNA-dependent RNA-polymerase complex (PB1, PB2, or PA; all approximately 79–83 kDa), the cleaved form of HA (HA1; approximately 61 kDa), and the Matrix protein (M1; approximately 21 kDa). This antibody also detects a nonspecific protein, which is also present in non-infected cells. Figure 1B shows a representative Western blot using A549 whole-cell lysates, which indicates that exposure to DE<sub>as</sub> increases viral protein levels. Similar to previously published reports, not all influenza virus proteins are detectable using this method (Nencioni *et al.*, 2003).

Enhanced levels of influenza virus replication could be caused by either increased viral production in each infected cell or increasing the number of infected cells. To analyze whether the increased levels of HA RNA and viral protein levels in DE<sub>as</sub>-exposed cells stems from increasing viral replication in each infected cell or from increasing the number of influenza-infected cells, we examined the effects of DE<sub>as</sub> exposures on the number of influenza-infected cells immunohistochemically. A549 cells were grown in chamber slides, exposed to DE<sub>as</sub>, and infected with three doses of influenza. Twenty-four h post-infection, cells were acetone-fixed and stained using a FITC-tagged anti-influenza antibody. The number of influenza-infected cells was examined using an epifluorescent microscope. Figure 1C shows that, independent of the dose of

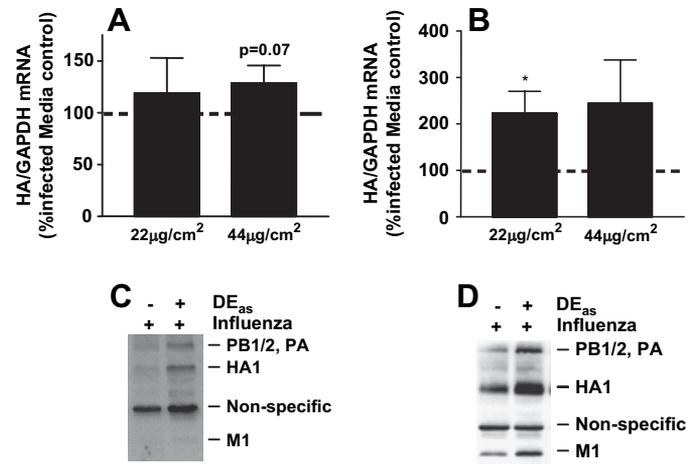
influenza used for infection, exposure to DE<sub>as</sub> increases the number of influenza-infected epithelial cells. In addition, the number of fluorescently stained cells in the control and DE<sub>as</sub>-exposed cells that were infected with 256 HAU influenza virus were counted. Figure 1D shows that DE<sub>as</sub> exposure significantly increases the number of influenza-infected A549 cells. Taken together, these results demonstrate that exposure to DE<sub>as</sub> increases the susceptibility of respiratory epithelial cells to become infected with influenza virus, resulting in a greater number of infected cells.

#### Exposure to DE<sub>as</sub> Increases the Susceptibility to Influenza Infections in Differentiated Human Respiratory Epithelial Cells

Primary human nasal and bronchial epithelial cells obtained from healthy human volunteers grown under defined culture conditions differentiate into a mucociliary phenotype, resembling many characteristics of human epithelium found *in vivo*, such as large beds of beating cilia and the presence of different cell types, including ciliated, nonciliated, and mucus-producing cells (Clark *et al.*, 1995). We used these models of differentiated human nasal and bronchial epithelial cells to confirm our findings obtained with A549 cells. Briefly, cultures of differentiated human nasal and bronchial epithelial cells were exposed to DE<sub>as</sub> for 2 h and subsequently infected with influenza A. Similar to our experiments using A549 cells, we analyzed viral RNA and protein levels in control and DE<sub>as</sub>-exposed cells 24 h post-infection. Figure 2 shows that exposure to DE<sub>as</sub> increased HA RNA levels in differentiated bronchial epithelial cells (Figure 2A) and differentiated nasal epithelial cells (Figure 2B), and that this difference was statistically significant for the nasal epithelial cells exposed to 22  $\mu\text{g}/\text{cm}^2$  and was approaching statistical significance for bronchial epithelial cells exposed to 44  $\mu\text{g}/\text{cm}^2$  ( $p = 0.07$ ). Figures 2C (bronchial epithelial cells) and 2D (nasal epithelial cells) also show that exposure of these cells to 44  $\mu\text{g}/\text{cm}^2$  DE<sub>as</sub> enhanced viral protein levels. Taken together, these results suggest that, similar to A549 cells, exposure to DE<sub>as</sub> also increases the susceptibility to influenza infection in cultures of primary differentiated human respiratory epithelial cells.

#### Effects of DE<sub>as</sub> Exposure on Influenza-Induced IFN Responses in A549 Cells

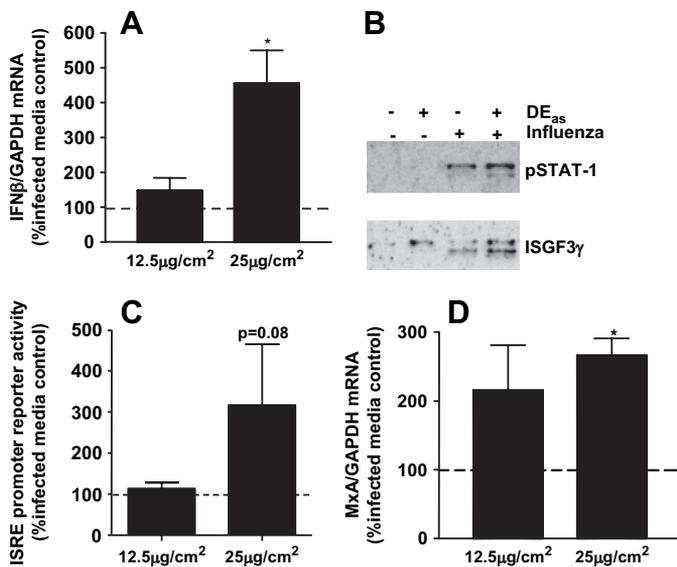
Previous studies conducted in mice have shown that chronic exposure to DE enhances influenza virus replication and that this effect was most likely caused by DE-induced suppression of interferon (IFN) levels (Hahon, *et al.*, 1985). Moreover, other *in vitro* and *in vivo* studies have demonstrated that exposure to DE can suppress basal or stimulus-induced IFN $\gamma$  production in immune cells (Saito *et al.*, 2002b; Yin *et al.*, 2004). While monocytes and other immune cells produce type II IFN, such as IFN $\gamma$ , respiratory epithelial cells only produce type I IFNs, which are predominantly IFN $\beta$  and IFN $\alpha$ .



**FIG. 2.** Exposure to DE<sub>as</sub> enhances susceptibility to influenza virus infections levels in differentiated nasal bronchial and bronchial epithelial cells. Cultures of differentiated human epithelial cells were exposed from the apical side to 22 or 44  $\mu\text{g}/\text{cm}^2$  DE<sub>as</sub> for 2 h and infected with influenza A/Bangkok/1/79. At 24 h post-infection cells were analyzed for influenza HA RNA levels by real-time RT-PCR. HA RNA levels were normalized to GAPDH mRNA levels and expressed as percent of the respective influenza-infected nonexposed media control. HA RNA levels in (A) differentiated human bronchial epithelial cells and (B) differentiated human nasal epithelial cells. Data are expressed as mean  $\pm$  SEM. \*Significantly different from the influenza-infected nonexposed media control;  $p > 0.05$ . Whole cell lysates of differentiated human epithelial cells exposed to 44  $\mu\text{g}/\text{cm}^2$  DE<sub>as</sub> for 2 h and infected with influenza A were analyzed for influenza virus proteins using an influenza A H3N2 serotype antibody at 24 h post-infection. Representative immunoblots from (C) differentiated human bronchial epithelial cells and (D) differentiated human nasal epithelial cells are shown.

Therefore, we determined the effects of DE<sub>as</sub> on influenza-induced IFN $\beta$  levels in human respiratory epithelial cells. Figure 3A shows that exposure of A549 cells to DE<sub>as</sub> does not suppress IFN $\beta$  mRNA levels. Indeed, Figure 3A indicates that treatment with DE<sub>as</sub> enhances influenza-induced IFN $\beta$  levels. Moreover, the effects of DE<sub>as</sub> on influenza-induced IFN $\beta$  levels correlate with the effects of DE<sub>as</sub> on influenza virus replication (compare Figure 3A with 1A).

Once IFN $\beta$  is produced, it acts in an autocrine or paracrine fashion to induce the synthesis of numerous IFN-stimulated genes (ISGs), which attempt to turn off viral infection in already infected cells or protect noninfected neighboring cells from becoming infected with the virus. Interaction of IFN $\beta$  with its specific receptors causes dimerization and activation of the receptors, which results in the recruitment of tyrosine kinases (Jak-1, Jak-2, and Tyk-2) (Samuel, 2001), and phosphorylation of the transcription factors STAT-1 and STAT-2. Phosphorylated and dimerized STAT-1/STAT-2, along with interferon-stimulated gene factor 3 gamma (ISGF3 $\gamma$ ) translocate into the nucleus and constitute a complex called ISGF-3, which binds to the *cis*-acting DNA element called ISRE (interferon specific response element), which in turn regulates the expression of numerous IFN-inducible genes. While



**FIG. 3.** Exposure to DE<sub>as</sub> enhances influenza-induced IFN responses in A549 cells. (A) A549 cells exposed to 12.5 or 25 μg/cm<sup>2</sup> DE<sub>as</sub> for 2 h and infected with influenza A were analyzed for IFNβ mRNA levels using real-time RT-PCR at 24 h post-infection. (B) A549 cells exposed to 25 μg/cm<sup>2</sup> DE<sub>as</sub> for 2 h and infected with influenza A were analyzed for nuclear phospho-STAT1 and ISGF3γ levels. Representative immunoblots for nuclear phospho-STAT1 (top) and ISGF3γ (bottom). (C) A549 cells were cotransfected with pISRE-luc and pSV-β-gal and exposed to 12.5 and 25 μg/cm<sup>2</sup> DE<sub>as</sub> and infected with influenza virus. At 24 h post-infection, specific luciferase activity in cell lysates was determined using β-gal activity as a normalizing factor. (D) A549 cells exposed to 12.5 or 25 μg/cm<sup>2</sup> DE<sub>as</sub> for 2 h and infected with influenza A were analyzed for MxA mRNA levels at 24 h post-infection using real-time RT-PCR. All mRNA levels were normalized to GAPDH mRNA levels and expressed as percent of the respective influenza-infected nonexposed media control. Data are expressed as mean ± SEM. \*Significantly different from the influenza-infected nonexposed media control; *p* > 0.05.

Figure 3A demonstrated that treatment with DE<sub>as</sub> also enhanced IFNβ mRNA levels, we determined whether IFN-dependent signaling was compromised by exposure to DE<sub>as</sub> and therefore suppressed the expression of ISGs, which are essential in the antiviral defense response. A549 cells were exposed to DE<sub>as</sub>, infected with influenza, and analyzed 24 h post-infection for nuclear levels of phospho-STAT-1 and ISGF3γ. As shown in Figure 3B, exposure to DE<sub>as</sub> enhances influenza-induced nuclear levels of both phospho-STAT-1 and ISGF3γ. Furthermore, Figure 3C shows that influenza-induced ISRE-promoter reporter activity was also enhanced by DE<sub>as</sub> exposure, albeit statistical significance was not reached. Together these data indicate that IFN-dependent signaling was not inhibited by treatment with DE<sub>as</sub>, but that activation of STAT-1 and ISGF3γ and ISRE-promoter reporter activity correlated well with the IFNβ levels in DE<sub>as</sub>-exposed and influenza-infected A549 cells.

Among the IFN-inducible antiviral mediators that are important in fighting influenza infections is myxovirus resistance protein (MxA). The importance of MxA in influenza infection is demonstrated by studies showing that cells

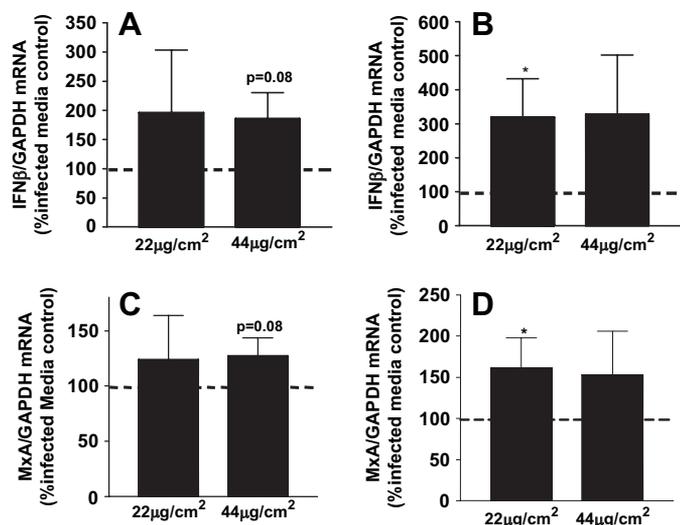
constitutively expressing MxA are resistant to influenza infections (Horisberger, 1995). The expression of MxA is ISRE dependent, and although our data indicated that exposure to DE<sub>as</sub> did not suppress activation of STAT-1 or ISGF3γ or ISRE-dependent promoter reporter activity, it is conceivable that DE<sub>as</sub> could affect the expression of essential antiviral mediators through different mechanisms. Therefore, we determined the effects of exposure to DE<sub>as</sub> on influenza-induced expression of MxA in A549 cells. Figure 3D demonstrates that the effect of DE<sub>as</sub> on influenza-induced MxA expression closely reflects the effects of DE<sub>as</sub> on HA RNA and IFNβ mRNA levels. Specifically, exposure of A549 cells to DE<sub>as</sub> enhanced MxA mRNA levels in these cells, indicating the DE<sub>as</sub> exposure does not compromise the ability of human respiratory epithelial cells to express antiviral mediators. While suppression of interferon-dependent antiviral defense responses would increase the susceptibility to influenza infections and reinfection of neighboring cells, increased interferon levels in the presence of increased viral replication would suggest that the interferon-dependent antiviral defense responses are working in accordance with a greater initial infection. Taken together, the data suggest that DE<sub>as</sub> enhances influenza virus replication without suppressing the production of IFNβ or IFNβ-inducible genes, such as MxA.

#### *Effects of DE<sub>as</sub> Exposure on Influenza-Induced IFN Responses in Differentiated Human Respiratory Epithelial Cells*

Again, to confirm our results on the effects of DE<sub>as</sub> exposure on influenza-induced IFN responses in primary epithelial cells, we exposed differentiated human nasal and bronchial epithelial cells to DE<sub>as</sub> and subsequently infected them with influenza A. Similar to experiments conducted in A549 cells, we analyzed the expression of IFNβ and MxA 24 h post-infection. Figures 4A and 4B show that, analogous to the effects seen in A549 cells, exposure to DE<sub>as</sub> also increases influenza-induced IFNβ mRNA levels in differentiated human bronchial (Fig. 4A) and nasal (Fig. 4B) epithelial cells. Moreover, exposure to DE<sub>as</sub> also increases influenza-induced MxA mRNA levels in differentiated human bronchial (Fig. 4C) and nasal (Fig. 4D) epithelial cells. Thus, similar to the effects of DE<sub>as</sub> on influenza-induced IFN responses seen in A549 cells, exposure to DE<sub>as</sub> also increases influenza-induced IFNβ and MxA mRNA levels in differentiated human nasal and bronchial epithelial cells. Again, these data suggest that the increased susceptibility to influenza infection seen after DE<sub>as</sub> exposure is not caused by impaired production of antiviral defense mediators.

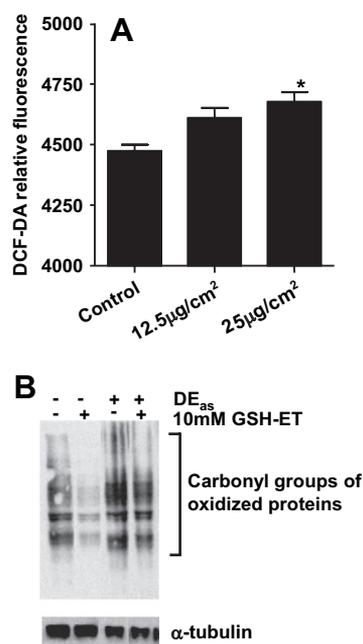
#### *Role of Oxidative Stress in the Effects of DE<sub>as</sub> on Influenza Infections*

Several studies have demonstrated that exposure to DE increases markers of oxidative stress and that many of the adverse effects associated with exposure to DE are mediated by oxidative stress (Hiura, *et al.*, 1999; Li *et al.*, 2002; Marano



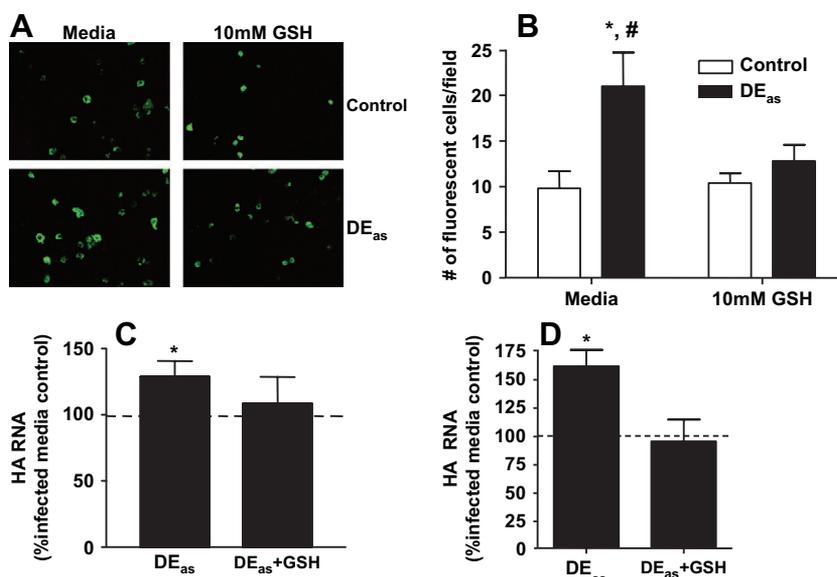
**FIG. 4.** Exposure to DE<sub>as</sub> enhances influenza-induced IFN responses in differentiated nasal bronchial and bronchial epithelial cells. Cultures of differentiated human epithelial cells were exposed from the apical side to 22 or 44 μg/cm<sup>2</sup> DE<sub>as</sub> for 2 h and infected with influenza A. At 24 h post-infection cells were analyzed for IFNβ and MxA mRNA levels by real-time RT-PCR. All mRNA levels were normalized to GAPDH mRNA levels and expressed as percent of the respective influenza-infected nonexposed control. IFNβ mRNA levels in (A) differentiated human bronchial epithelial cells and (B) differentiated human nasal epithelial cells; MxA mRNA levels in (C) differentiated human bronchial epithelial cells and (D) differentiated human nasal epithelial cells. Data are expressed as mean ± SEM. \*Significantly different from the influenza-infected nonexposed media control;  $p > 0.05$ .

*et al.*, 2002). However, the extent to which DE can induce oxidative stress may depend on the type and amount of polyaromatic hydrocarbons (PAH) adsorbed to the surface of the diesel particles (Bonvallot *et al.*, 2001). This in turn depends on the type of diesel fuel, the diesel engine, and the engine load during particle collection (Madden, *et al.*, 2003). Therefore, we determined oxidative stress induced in respiratory epithelial cells after exposure to the DE<sub>as</sub> used in this study. We analyzed DE<sub>as</sub>-induced oxidative stress by measuring the oxidation of the nonfluorescent 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA), to the highly fluorescent 2',7'-dichlorofluorescein (DCF) as described before (Jaspers, *et al.*, 2000). Figure 5A shows that exposure to DE<sub>as</sub> dose-dependently increases oxidative stress in A549 cells within 2 h post-exposure. In addition, we determined protein carbonyl levels in A549 cells, a marker of post-transcriptional oxidative modification of cellular proteins. Figure 5B shows that exposure to DE<sub>as</sub> enhances protein carbonyl levels and that prior addition of 10 mM GSH-ET, a cell-permeable form of the antioxidant GSH, decreased the levels of protein carbonyls. The levels of GSH-ET used here (10 mM) enhance cellular GSH levels (data not shown). Therefore, these data demonstrate that exposure to DE<sub>as</sub> increases markers of oxidative stress and that increasing cellular GSH levels by adding the antioxidant GSH-ET reverses this effect.



**FIG. 5.** Exposure to DE<sub>as</sub> enhances markers of oxidative stress in A549 cells. (A) A549 cells were loaded with 10 μM DCF-DA for 30 min before exposure to 0 (control), 12.5, or 25 μg/cm<sup>2</sup> DE<sub>as</sub>. Fluorescence was measured 2 h post-exposure using a fluorescence plate reader. Data are expressed as mean ± SEM. \*Significantly different from control;  $p > 0.05$ . (B) A549 cells were treated with 10 mM GSH-ET 30 min before exposure to 25 μg/cm<sup>2</sup> DE<sub>as</sub>. At 24 h post infection, whole cell lysates were analyzed for the presence of oxidized protein carbonyl levels by SDS-PAGE using OxyBlot™. Nitrocellulose membranes were stripped and re-probed with anti-α-tubulin antibody to assure equal loading. Representative immunoblots are shown.

Based on the evidence shown above, we then examined the role of DE<sub>as</sub>-induced oxidative stress in the enhanced susceptibility to influenza virus infections in these cells. A549 cells were treated with 10 mM GSH-ET 30 min before DE<sub>as</sub> exposure and subsequently infected with influenza virus. The effects of treatment with GSH-ET on influenza infections were analyzed by assessing the number of influenza-infected cells and by measuring HA RNA levels. Figure 6A shows that exposure to DE<sub>as</sub> increases the number of influenza-infected cells and that pretreatment with 10 mM GSH-ET reverses the effects of DE<sub>as</sub> on the number of influenza-infected cells. The number of influenza-infected cells was counted in six separate microscopic fields, and the data were summarized in Figure 6B, which demonstrates that, while DE<sub>as</sub> exposure alone significantly increases the number of influenza-infected cells, pretreatment with GSH-ET reverses this number to nonexposed control levels. Similar to the effects of GSH-ET on number of influenza-infected cells, treatment with GSH-ET also reverses the effects of DE<sub>as</sub> on HA RNA levels in A549 cells (Fig. 6C). To confirm the role of oxidative stress in the effects of DE<sub>as</sub> on the susceptibility to influenza infection in primary human epithelial cells, differentiated human bronchial epithelial cells were also pretreated with 10 mM GSH-ET, exposed to DE<sub>as</sub>,



**FIG. 6.** Addition of GSH reverses effects of  $DE_{as}$  on influenza infection in respiratory epithelial cells. (A) A549 cells were treated with 10 mM GSH-ET 30 min before exposure to  $25 \mu\text{g}/\text{cm}^2$   $DE_{as}$  and infection with influenza A. At 24 h post-infection, cells were acetone-fixed and stained using FITC-tagged anti-influenza A antibody. Representative image shows the effects of  $DE_{as}$  in the presence or absence of GSH on direct immunofluorescent localization of influenza-infected cells. (B) Average number of immunofluorescent cells counted in six different microscopic fields from A549 cells infected with influenza A after exposure to  $DE_{as}$  in the presence or absence of GSH. Data are expressed as mean  $\pm$  SEM. \*Significantly different from  $DE_{as}$ -exposed and GSH-treated cells, #significantly different from nonexposed and not GSH-treated cells; (C) A549 cells or (D) differentiated bronchial epithelial cells were supplemented with 10 mM GSH-ET 30 min prior to exposure to  $25 \mu\text{g}/\text{cm}^2$  (A549 cells) or  $44 \mu\text{g}/\text{cm}^2$  (differentiated bronchial epithelial cells)  $DE_{as}$ . Two h after  $DE_{as}$  exposure cells were infected with influenza A/Bangkok/1/79. At 24 h post infection, total RNA was analyzed for influenza virus HA RNA levels using real-time RT-PCR. HA RNA levels were normalized to GAPDH mRNA levels and expressed as percent of the respective influenza-infected nonexposed media control. Data are expressed as mean  $\pm$  SEM. \*Significantly different from the respective influenza-infected nonexposed media control;  $p > 0.05$ .

and infected with influenza A. Similar to the results seen in A549 cells, Figure 6D shows that pretreatment with GSH-ET also reversed the effects of  $DE_{as}$  on HA RNA levels in differentiated bronchial epithelial cells. Thus, oxidative stress induced upon exposure to  $DE_{as}$  is at least partially responsible for increasing the susceptibility of respiratory epithelial cells to influenza infection.

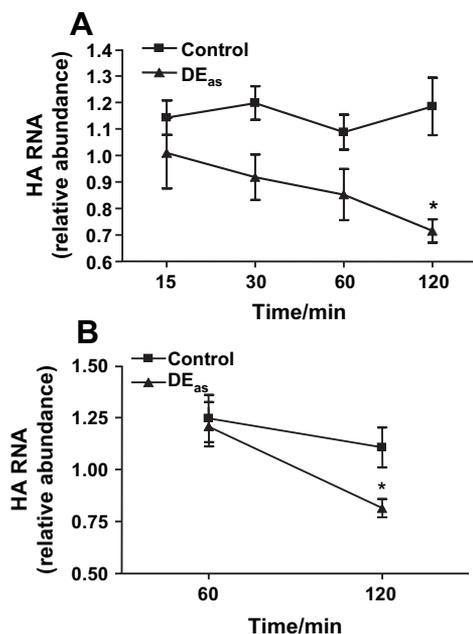
#### Effects of $DE_{as}$ on Influenza Virus Attachment

Our data thus far have shown that exposure to  $DE_{as}$  enhances the number of influenza-infected cells and influenza virus replication without decreasing IFN-dependent antiviral defense responses. Taken together, these data suggest that exposure to  $DE_{as}$  increases the susceptibility to influenza infections by enhancing the ability of influenza virus to attach, enter, and infect epithelial cells. To determine the effects of  $DE_{as}$  exposure on influenza virus attachment and entry, we conducted virus attachment assays. Briefly, control and  $DE_{as}$ -exposed A549 cells were infected with influenza virus, and immediately, or 15, 30, 60, and 120 min after addition of the virus, the nonattached virus was removed by rinsing the cells twice with media. The level of nonattached virus was determined by isolating viral RNA from these rinses and analyzing them for HA RNA levels by quantitative RT-PCR, as a measure of the number of nonattached virus particles.

Decreased HA RNA levels in the rinses would indicate lower number of virus particles that did not attach and therefore indicate increased attachment and uptake of the virus by the epithelial cells. Figure 7A demonstrates that exposure to  $DE_{as}$  significantly increases attachment of influenza virus to A549 cells within 2 h post-infection. To confirm these results in primary epithelial cells, we conducted the same experiment in differentiated nasal epithelial cells. Briefly, control and  $DE_{as}$ -exposed differentiated nasal epithelial cells were infected with influenza virus, and immediately, 60, or 120 min after addition the levels of nonattached viruses were analyzed by real-time RT-PCR. Figure 7B shows that, similar to the results seen in A549 cells, exposure to  $DE_{as}$  also increases influenza virus attachment and entry into differentiated human nasal epithelial cells.

## DISCUSSION

Previous studies have demonstrated that repeated exposures of mice to DE increase their susceptibility to respiratory virus infections (Hahon *et al.*, 1985; Harrod *et al.*, 2003). Based on these findings we hypothesized that similar findings could be observed *in vitro* using models of human respiratory epithelium and that *in vitro* studies could be used to discern potential mechanisms mediating enhanced susceptibility to viral infections following air pollutant exposures. The results presented



**FIG. 7.** Exposure to DE<sub>as</sub> increases influenza virus attachment to respiratory epithelial cells. (A) A549 cells or (B) differentiated nasal epithelial cells were exposed to DE<sub>as</sub> for 2 h. Influenza virus was added to the cells and immediately ( $t = 0$ ) or at 15, 30, 60, and 120 min after addition of the virus nonattached virus was removed from the cells. The amount of nonattached virus was analyzed by measuring HA RNA levels by real-time RT-PCR. HA RNA levels in control and DE<sub>as</sub>-exposed cells were normalized to the HA RNA levels recovered immediately after addition of the virus in the respective exposure group. The data indicate that in both cell types exposure to DE<sub>as</sub> decreases the level of nonattached virus remaining at 2 h post-infection, indicating greater virus attachment in these cells. Data are expressed as mean  $\pm$  SEM. \*Significantly different from influenza-infected non-exposed media control;  $p > 0.05$ .

here demonstrate that acute exposure to DE<sub>as</sub> enhances the susceptibility to influenza infections, not by suppressing IFN-dependent antiviral defense responses, but by enhancing virus attachment and possibly uptake by respiratory epithelial cells. Enhanced susceptibility to influenza virus infection was mediated by DE<sub>as</sub>-induced oxidative stress and was observed in differentiated human bronchial and nasal epithelial cells as well as A549 cells, a human cell line with type II-cell like characteristics (Nardone *et al.*, 1979). Thus, the effects of DE<sub>as</sub> exposure on influenza infections were observed in a respiratory epithelial cell line as well as primary differentiated human respiratory epithelial cells derived from the upper and lower respiratory tract. All of these different *in vitro* models of the human respiratory epithelium represent cells that can support replication of influenza virus, are cell types potentially affected by inhaled DE, and are therefore the potential site where interactions between influenza infection and DE could occur *in vivo*. Although there have been a few studies examining the effects of DE exposures on subsequent influenza infections in mice (Castranova *et al.*, 2001; Hahon *et al.*, 1985; Rabovsky *et al.*, 1986), at least to our knowledge, the results presented

here are the first evidence suggesting that such interactions could adversely affect influenza infections in human cells. Considering the number of people infected with influenza virus each year and the population routinely exposed to DE, these data could have significant public health impact.

The enhanced susceptibility to influenza virus infections after DE<sub>as</sub> exposures in our *in vitro* models was not caused by suppression of IFN $\beta$  or IFN-dependent mediator production. In fact, our data demonstrated that the levels of IFN $\beta$ , IFN $\beta$ -dependent signaling, and MxA expression correlated well with the levels of influenza virus replication. Enhanced IFN production in the presence of enhanced influenza virus replication would suggest that the ability to generate IFN-dependent antiviral defense responses was not impaired by DE<sub>as</sub> exposure, but that the host tissue was responding appropriately to a greater level of viral infection. Indeed, our data demonstrated that the number of influenza-infected cells was enhanced by DE<sub>as</sub> exposures, which would explain the increased levels of IFN and IFN-dependent antiviral defense response. On the other hand, suppressed IFN production in the presence of enhanced influenza virus replication would suggest that the host cells are incapable of limiting the viral infection, which enhances replication and reinfection, thus increasing the spreading of the infection. Previously published reports demonstrated that chronic exposure to DE enhanced influenza virus replication and decreased lung IFN levels (Hahon *et al.*, 1985), suggesting that the ability of the host to limit and clear the infection was impaired by chronic DE exposures. In contrast, subchronic exposure to DE enhanced the susceptibility to infection with respiratory syncytial virus (RSV) without impairing IFN production (Harrod *et al.*, 2003). These studies suggest that chronic and subchronic exposures to DE may increase the susceptibility to respiratory virus infections through IFN-dependent and IFN-independent mechanisms, respectively. Our studies only examined the effects of acute DE<sub>as</sub> exposures on influenza infections in epithelial cells *in vitro*, and since epithelial cells only produce type I interferons, we focused on the effects of DE<sub>as</sub> on influenza-induced IFN $\beta$  production. However, it is conceivable that acute exposures to DE<sub>as</sub> could impair influenza-induced IFN $\gamma$  production, a type II interferon, by infected monocytes, macrophages, or other immune cells.

However, other non-IFN-dependent antiviral defense strategies applied by respiratory epithelial cells to limit influenza infection may have been affected by acute and subchronic exposures to DE<sub>as</sub>. For example, studies by Harrod *et al.* (2003) have demonstrated that subchronic exposures to DE enhances RSV titers in mice and that this effect was associated with decreased levels of other mediators with potential antiviral function. Specifically, this study demonstrated that exposure to DE decreased lung surfactant protein A (SP-A) and Clara cell secretory protein (CCSP) levels, which can both have RSV-scavenging activity (Harrod *et al.*, 1998, 1999). SP-A as well as surfactant protein D (SP-D) both belong to the family of collectins, which recognize and interact with glycoconjugates

on the surface of microorganisms and thus increase clearance of the microorganisms by phagocytic cells (Wright, 2004). Both SP-A and SP-D are released by alveolar type II cells as well as nonciliated bronchial epithelial cells or Clara cells (Madsen *et al.*, 2000, 2003) and are very important for innate immediate defense responses against influenza infections (Hartshorn *et al.*, 1994; Hawgood *et al.*, 2004). Specifically, SP-D and SP-A can agglutinate influenza virus, neutralize the virus, and therefore prevent attachment to the host cell (Hartshorn *et al.*, 2000; Hawgood *et al.*, 2004). Given the previous reports on the effects of DE exposures on SP-A levels in mice (Harrod *et al.*, 2003) and the fact that SP-A and SP-D are produced by alveolar type II cells and nonciliated bronchial epithelial cells, it is plausible that in our *in vitro* models of human respiratory epithelial cells acute exposures to DE<sub>as</sub> modified the ability of SP-D or SP-A to neutralize influenza virus in these cells.

Our data show that DE<sub>as</sub>-induced oxidative stress increased the susceptibility to influenza infection in human respiratory epithelial cells and that DE<sub>as</sub> exposure increased markers of oxidative stress within 2 h post-exposure. In our experimental model, respiratory epithelial cells were exposed to DE<sub>as</sub> 2 h prior to infection with influenza, suggesting that, at the time of infection, DE<sub>as</sub>-exposed cells were already oxidatively stressed. Previous studies have shown that influenza virus replication *in vitro* and *in vivo* was inhibited by the antioxidant GSH (Cai *et al.*, 2003), suggesting that oxidative stress would favor influenza virus replication. The mechanisms by which oxidative stress could increase the susceptibility to influenza infections are not clear. Based on our observations that DE<sub>as</sub> exposure enhances influenza virus attachment and entry within 2 h post-infection, it seems likely that redox-dependent post-translational modification of components regulating susceptibility to influenza virus infections could be involved. For example, influenza virus hemagglutinin needs to be proteolytically cleaved for the virus to become infectious and enter the cells, which is mediated by trypsin-like serine proteases released by epithelial cells (Kido *et al.*, 1992; Sakai *et al.*, 1993). The activity of such proteases in turn is regulated by mucus antiproteases, such as secretory leukocyte protease inhibitor (SLPI) (Kido *et al.*, 1999). Interestingly, oxidative stress derived from cigarette smoke or addition of reactive oxygen intermediates can decrease antiprotease activity (Cavarra *et al.*, 2001; Vogelmeier *et al.*, 1997) and increase influenza infectivity (Hennet *et al.*, 1992). Thus, post-translational modification of antiproteases by DE<sub>as</sub>-induced oxidative stress could increase the ability to proteolytically activate influenza virus and therefore enhance the susceptibility to influenza infections.

In addition, the activity of collectins, specifically SP-A, is sensitive to oxidative modifications. Wang *et al.* (2002) have shown that oxidative modification of SP-A after ozone exposure alters the ability of SP-A to stimulate cytokine production in a monocytic cell line. As stated above, previous

studies have shown that subchronic exposures to DE can decrease the transcriptional levels of SP-A in mice, which may have contributed to the increase susceptibility to RSV infections in these animals (Harrod *et al.*, 2003). However, the results presented here showed increased influenza virus attachment after only a brief exposure (2 h) to DE<sub>as</sub>, suggesting that the transcriptional levels of SP-A or other collectins may not have changed during this short exposure period. Alternatively, it is also possible that post-translational oxidative modification of SP-A or other collectins reduced the ability to aggregate and neutralize influenza virus and therefore increased susceptibility to infection. Indeed, post-translational nitration of SP-A through reactive nitrogen species has been demonstrated to decrease the ability of SP-A to aggregate lipids, bind mannose, and adhere *P. carinii* to macrophages (Haddad *et al.*, 1996; Zhu *et al.*, 1996, 1998), all of which are important functions of SP-A *in vivo*. Thus, in addition to modifying the transcriptional level of SP-A and other innate immune defense mediators, oxidative stress induced upon exposure to DE could also reduce the function of SP-A and other collectins through post-translational oxidation of these proteins. Future studies will examine the effects of DE<sub>as</sub> and oxidative modification of SP-D on its ability to neutralize influenza *in vitro*.

All of the experiments described in this study were performed *in vitro* using differentiated primary human respiratory epithelial cells or a human lung carcinoma cell line (A549 cells). Interestingly, the primary cells and the cell line responded very similarly to the treatment with DE<sub>as</sub> and influenza infection, although the responses did not always reach statistical significance for the bronchial epithelial cells. Differentiated primary human respiratory epithelial cells, when grown under defined culture condition (see Methods), retain many characteristics seen *in vivo* (Clark *et al.*, 1995; Gray *et al.*, 1996; Ostrowski *et al.*, 1995). Interestingly, our data indicate that differentiated human nasal epithelial cells may be more sensitive to the effects of DE<sub>as</sub> on influenza virus replication than human bronchial epithelial cells. This becomes even more important, considering the nasal epithelium as the site where a large fraction of DE particles are potentially deposited during inhalation and exhalation (Wiesmiller *et al.*, 2003) and as the site of initial infection with influenza virus. However, the role of the respiratory epithelium during influenza infections goes beyond viral replication and innate immune defense responses. Respiratory epithelial cells produce and release a number of pro-inflammatory cytokines and chemokines in response to influenza infections (Adachi *et al.*, 1997; Julkunen *et al.*, 2000), which are responsible for recruitment and activation of mononuclear cells, costimulation of T<sub>h</sub> lymphocytes, induction of lymphocyte/fibroblast proliferation, acting as endogenous pyrogens, and other activities. Thus, the respiratory epithelium is not only an entryway for influenza infections, but also a key element which orchestrates many of the subsequent immune and inflammatory responses.

Consequently, enhanced susceptibility of respiratory epithelial cells to influenza infections after exposure to DE will determine the severity of the infection and injury to the surrounding tissue.

Taken together, the data presented here demonstrate that acute exposures to DE<sub>as</sub> significantly affect the susceptibility of human respiratory epithelial cells to influenza virus infections by enhancing virus attachment and entry. The aqueous-trapped solution of DE used throughout these studies contains both the particulate fraction and water-soluble gas-phase components of DE derived from an off-road diesel engine. In both differentiated human epithelial cells and A549 cells, the doses of DE<sub>as</sub> used throughout the study did not cause cytotoxicity (data not shown), suggesting that DE<sub>as</sub> did not significantly affect the integrity of the epithelial layer. Future studies will determine whether different preparations of DE particles as well as other ambient air pollution particles have similar effects on influenza infections in respiratory epithelial cells. Concurrent exposures to DE or other PM mixtures and influenza infections are likely in urban populations. Therefore, enhanced susceptibility to influenza infections resulting from DE or PM mixtures could have a significant impact on public health.

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#### REFERENCES

- Adachi, M., Matsukura, S., Tokunaga, H., and Kokubu, F. (1997). Expression of cytokines on human bronchial epithelial cells induced by influenza virus A. *Int. Arch. Allergy Immunol.* **113**, 307–311.
- Beck, M. A., and Matthews, C. C. (2000). Micronutrients and host resistance to viral infection. *Proc. Nutr. Soc.* **59**, 581–585.
- Beck, M. A., Nelson, H. K., Shi, Q., Van Dael, P., Schiffrin, E. J., Blum, S., Barclay, D., and Levander, O. A. (2001). Selenium deficiency increases the pathology of an influenza virus infection. *FASEB J.* **15**, 1481–1483.
- Bonvallot, V., Baeza-Squiban, A., Baulig, A., Brulant, S., Boland, S., Muzeau, F., Barouki, R., and Marano, F. (2001). Organic compounds from diesel exhaust particles elicit a proinflammatory response in human airway epithelial cells and induce cytochrome p450 1A1 expression. *Am. J. Respir. Cell Mol. Biol.* **25**, 515–521.
- Cai, J., Chen, Y., Seth, S., Furukawa, S., Compans, R. W., and Jones, D. P. (2003). Inhibition of influenza infection by glutathione. *Free Radic. Biol. Med.* **34**, 928–936.
- Castranova, V., Ma, J. Y., Yang, H. M., Antonini, J. M., Butterworth, L., Barger, M. W., Roberts, J., and Ma, J. K. (2001). Effect of exposure to diesel exhaust particles on the susceptibility of the lung to infection. *Environ. Health Perspect.* **109** (Suppl. 4), 609–612.
- Cavarra, E., Lucattelli, M., Gambelli, F., Bartalesi, B., Fineschi, S., Szarka, A., Giannerini, F., Martorana, P. A., and Lungarella, G. (2001). Human SLPI inactivation after cigarette smoke exposure in a new *in vivo* model of pulmonary oxidative stress. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **281**, L412–L417.
- Clark, A. B., Randell, S. H., Nettesheim, P., Gray, T. E., Bagnell, B., and Ostrowski, L. E. (1995). Regulation of ciliated cell differentiation in cultures of rat tracheal epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **12**, 329–338.
- D'Amato, G. (2002). Urban air pollution and respiratory allergy. *Monaldi Arch. Chest Dis.* **57**, 136–140.
- Diaz-Sanchez, D. (1997). The role of diesel exhaust particles and their associated polyaromatic hydrocarbons in the induction of allergic airway disease. *Allergy* **52**, 52–56.
- Gray, T. E., Guzman, K., Davis, C. W., Abdullah, L. H., and Nettesheim, P. (1996). Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **14**, 104–112.
- Haddad, I. Y., Zhu, S., Ischiropoulos, H., and Matalon, S. (1996). Nitration of surfactant protein A results in decreased ability to aggregate lipids. *Am. J. Physiol.* **270**, L281–L288.
- Hahon, N., Booth, J. A., Green, F., and Lewis, T. R. (1985). Influenza virus infection in mice after exposure to coal dust and diesel engine emissions. *Environ. Res.* **37**, 44–60.
- Harrod, K. S., Jaramillo, R. J., Rosenberger, C. L., Wang, S. Z., Berger, J. A., McDonald, J. D., and Reed, M. D. (2003). Increased susceptibility to RSV infection by exposure to inhaled diesel engine emissions. *Am. J. Respir. Cell Mol. Biol.* **28**, 451–463.
- Harrod, K. S., Munday, A. D., Stripp, B. R., and Whitsett, J. A. (1998). Clara cell secretory protein decreases lung inflammation after acute virus infection. *Am. J. Physiol.* **275**, L924–L930.
- Harrod, K. S., Trapnell, B. C., Otake, K., Korfhagen, T. R., and Whitsett, J. A. (1999). SP-A enhances viral clearance and inhibits inflammation after pulmonary adenoviral infection. *Am J Physiol* **277**, L580–L588.
- Hartshorn, K. L., Crouch, E. C., White, M. R., Eggleton, P., Tauber, A. I., Chang, D., and Sastry, K. (1994). Evidence for a protective role of pulmonary surfactant protein D (SP-D) against influenza A viruses. *J. Clin. Invest.* **94**, 311–319.
- Hartshorn, K. L., White, M. R., Voelker, D. R., Coburn, J., Zaner, K., and Crouch, E. C. (2000). Mechanism of binding of surfactant protein D to influenza A viruses: Importance of binding to haemagglutinin to antiviral activity. *Biochem. J.* **351**(2), 449–458.
- Hashimoto, S., Gon, Y., Takeshita, I., Matsumoto, K., Jibiki, I., Takizawa, H., Kudoh, S., and Horie, T. (2000). Diesel exhaust particles activate p38 MAP kinase to produce interleukin 8 and RANTES by human bronchial epithelial cells and N-acetylcysteine attenuates p38 MAP kinase activation. *Am. J. Respir. Crit. Care Med.* **161**, 280–285.
- Hawgood, S., Brown, C., Edmondson, J., Stumbaugh, A., Allen, L., Goerke, J., Clark, H., and Poulain, F. (2004). Pulmonary collectins modulate strain-specific influenza A virus infection and host responses. *J. Virol.* **78**, 8565–8572.
- Hennet, T., Peterhans, E., and Stocker, R. (1992). Alterations in antioxidant defences in lung and liver of mice infected with influenza A virus. *J. Gen. Virol.* **73**(1), 39–46.

- Hiura, T. S., Kaszubowski, M. P., Li, N., and Nel, A. E. (1999). Chemicals in diesel exhaust particles generate reactive oxygen radicals and induce apoptosis in macrophages. *J. Immunol.* **163**, 5582–5591.
- Hiura, T. S., Li, N., Kaplan, R., Horwitz, M., Seagrave, J. C., and Nel, A. E. (2000). The role of a mitochondrial pathway in the induction of apoptosis by chemicals extracted from diesel exhaust particles. *J. Immunol.* **165**, 2703–2711.
- Horisberger, M. A. (1995). Interferons, Mx genes, and resistance to influenza virus. *Am. J. Respir. Crit. Care Med.* **152**, S67–S71.
- Jaspers, I., Samet, J. M., Erzurum, S., and Reed, W. (2000). Vanadium-induced kB-dependent transcription depends upon peroxide-induced activation of the p38 mitogen-activated protein kinase. *Am. J. Resp. Cell Mol. Biol.* **23**, 95–102.
- Jaspers, I., Samet, J. M., and Reed, W. (1999). Arsenite Exposure of Cultured Airway Epithelial Cells Activates kB-dependent IL-8 Gene Expression in the Absence of NF-kB Nuclear Translocation. *J. Biol. Chem.* **274**(43), 31025–31033.
- Jaspers, I., Zhang, W., Fraser, A., Samet, J. M., and Reed, W. (2001). Hydrogen peroxide has opposing effects on IKK activity and proteasomal degradation of IκBa in airway epithelial cells. *Am. J. Resp. Cell Mol. Biol.* **24**, 769–777.
- Julkunen, I., Melen, K., Nyqvist, M., Pirhonen, J., Sareneva, T., and Matikainen, S. (2000). Inflammatory responses in influenza A virus infection. *Vaccine* **19** (Suppl. 1), S32–S37.
- Kido, H., Beppu, Y., Imamura, Y., Chen, Y., Murakami, M., Oba, K., and Towatari, T. (1999). The human mucus protease inhibitor and its mutants are novel defensive compounds against infection with influenza A and Sendai viruses. *Biopolymers* **51**, 79–86.
- Kido, H., Yokogoshi, Y., Sakai, K., Tashiro, M., Kishino, Y., Fukutomi, A., and Katunuma, N. (1992). Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A possible activator of the viral fusion glycoprotein. *J. Biol. Chem.* **267**, 13573–13579.
- LeVine, A. M., Gwozdz, J., Stark, J., Bruno, M., Whitsett, J., and Korfhagen, T. (1999). Surfactant protein-A enhances respiratory syncytial virus clearance *in vivo*. *J. Clin. Invest.* **103**, 1015–1021.
- Li, N., Wang, M., Oberley, T. D., Sempf, J. M., and Nel, A. E. (2002). Comparison of the pro-oxidative and proinflammatory effects of organic diesel exhaust particle chemicals in bronchial epithelial cells and macrophages. *J. Immunol.* **169**, 4531–4541.
- Madden, M. C., Dailey, L. A., Stonehurner, J. G., and Harris, B. D. (2003). Responses of cultured human airway epithelial cells treated with diesel exhaust extracts will vary with the engine load. *J. Toxicol. Environ. Health A* **66**, 2281–2297.
- Madsen, J., Kliem, A., Tornøe, I., Skjold, K., Koch, C., and Holmskov, U. (2000). Localization of lung surfactant protein D on mucosal surfaces in human tissues. *J. Immunol.* **164**, 5866–5870.
- Madsen, J., Tornøe, I., Nielsen, O., Koch, C., Steinhilber, W., and Holmskov, U. (2003). Expression and localization of lung surfactant protein A in human tissues. *Am. J. Respir. Cell Mol. Biol.* **29**, 591–597.
- Marano, F., Boland, S., Bonvallot, V., Baulig, A., and Baeza-Squiban, A. (2002). Human airway epithelial cells in culture for studying the molecular mechanisms of the inflammatory response triggered by diesel exhaust particles. *Cell Biol. Toxicol.* **18**, 315–320.
- Nardone, L. L., and Andrews, S. B. (1979). Cell line A549 as a model of the type II pneumocyte. Phospholipid biosynthesis from native and organometallic precursors. *Biochim. Biophys. Acta* **573**, 276–295.
- Nencioni, L., Iuvara, A., Aquilano, K., Ciriolo, M. R., Cozzolino, F., Rotilio, G., Garaci, E., and Palamara, A. T. (2003). Influenza A virus replication is dependent on an antioxidant pathway that involves GSH and Bcl-2. *FASEB J.* **17**, 758–760.
- Ostrowski, L. E., Randell, S. H., Clark, A. B., Gray, T. E., and Nettesheim (1995). Ciliogenesis of rat tracheal epithelial cells *in vitro*. *Methods Cell Biol.* **47**, 57–63.
- Pandya, R. J., Solomon, G., Kinner, A., and Balmes, J. R. (2002). Diesel exhaust and asthma: Hypotheses and molecular mechanisms of action. *Environ. Health Perspect.* **110** (Suppl. 1), 103–112.
- Rabovsky, J., Judy, D. J., Rodak, D. J., and Petersen, M. (1986). Influenza virus-induced alterations of cytochrome P-450 enzyme activities following exposure of mice to coal and diesel particulates. *Environ. Res.* **40**, 136–144.
- Saito, Y., Azuma, A., Kudo, S., Takizawa, H., and Sugawara, I. (2002a). Effects of diesel exhaust on murine alveolar macrophages and a macrophage cell line. *Exp. Lung Res.* **28**, 201–217.
- Saito, Y., Azuma, A., Kudo, S., Takizawa, H., and Sugawara, I. (2002b). Long-term inhalation of diesel exhaust affects cytokine expression in murine lung tissues: Comparison between low- and high-dose diesel exhaust exposure. *Exp. Lung Res.* **28**, 493–506.
- Sakai, K., Kawaguchi, Y., Kishino, Y., and Kido, H. (1993). Electron immunohistochemical localization in rat bronchiolar epithelial cells of tryptase Clara, which determines the pneumotropism and pathogenicity of Sendai virus and influenza virus. *J. Histochem. Cytochem.* **41**, 89–93.
- Samuel, C. E. (2001). Antiviral actions of interferons. *Clin. Microbiol. Rev.* **14**, 778–809.
- Spackman, E., Senne, D. A., Myers, T. J., Bulaga, L. L., Garber, L. P., Perdue, M. L., Lohman, K., Daum, L. T., and Suarez, D. L. (2002). Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J. Clin. Microbiol.* **40**, 3256–3260.
- Steenberg, P., Verlaan, A., De Klerk, A., Boere, A., Loveren, H., and Cassee, F. (2004). Sensitivity to ozone, diesel exhaust particles, and standardized ambient particulate matter in rats with a listeria monocytogenes-induced respiratory infection. *Inhal. Toxicol.* **16**, 311–317.
- Takizawa, H., Abe, S., Okazaki, H., Kohyama, T., Sugawara, I., Saito, Y., Ohtoshi, T., Kawasaki, S., Desaki, M., Nakahara, K., et al. (2003). Diesel exhaust particles upregulate eotaxin gene expression in human bronchial epithelial cells via nuclear factor-kappa B-dependent pathway. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **284**, L1055–L1062.
- Takizawa, H., Ohtoshi, T., Kawasaki, S., Abe, S., Sugawara, I., Nakahara, K., Matsushima, K., and Kudoh, S. (2000). Diesel exhaust particles activate human bronchial epithelial cells to express inflammatory mediators in the airways: A review. *Respirology* **5**, 197–203.
- Thompson, W. W., Shay, D. K., Weintraub, E., Brammer, L., Bridges, C. B., Cox, N. J., and Fukuda, K. (2004). Influenza-associated hospitalizations in the United States. *JAMA* **292**, 1333–1340.
- Thompson, W. W., Shay, D. K., Weintraub, E., Brammer, L., Cox, N., Anderson, L. J., and Fukuda, K. (2003). Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA* **289**, 179–186.
- U.S. EPA (2002). Health Assessment Document for Diesel Engine Exhaust. United States Environmental Protection Agency, EPA/600/8–90/057F.
- Vogelmeier, C., Biedermann, T., Maier, K., Mazur, G., Behr, J., Krombach, F., and Buhl, R. (1997). Comparative loss of activity of recombinant secretory leukoprotease inhibitor and alpha 1-protease inhibitor caused by different forms of oxidative stress. *Eur. Respir. J.* **10**, 2114–2119.
- Wang, G., Umstead, T. M., Phelps, D. S., Al-Mondhiry, H., and Floros, J. (2002). The effect of ozone exposure on the ability of human surfactant protein A variants to stimulate cytokine production. *Environ. Health Perspect.* **110**, 79–84.
- Wang, S. Z., Rosenberger, C. L., Bao, Y. X., Stark, J. M., and Harrod, K. S. (2003). Clara cell secretory protein modulates lung inflammatory and immune responses to respiratory syncytial virus infection. *J. Immunol.* **171**, 1051–1060.
- Wiesmiller, K., Keck, T., Leiacker, R., Sikora, T., Rettinger, G., and Lindemann, J. (2003). The impact of expiration on particle deposition within the nasal cavity. *Clin Otolaryngol.* **28**, 304–307.
- Wright, J. R. (2004). Host defense functions of pulmonary surfactant. *Biol. Neonate* **85**, 326–332.

- Wu, R., Sato, G. H., and Whitcutt, M. J. (1986). Developing differentiated epithelial cell cultures: Airway epithelial cells. [Review] [55 refs]. *Fundam. Appl. Toxicol.* **6**, 580–590.
- Yang, H. M., Antonini, J. M., Barger, M. W., Butterworth, L., Roberts, B. R., Ma, J. K., Castranova, V., and Ma, J. Y. (2001). Diesel exhaust particles suppress macrophage function and slow the pulmonary clearance of *Listeria monocytogenes* in rats. *Environ. Health Perspect.* **109**, 515–521.
- Yin, X. J., Dong, C. C., Ma, J. Y., Antonini, J. M., Roberts, J. R., Stanley, C. F., Schafer, R., and Ma, J. K. (2004). Suppression of Cell-Mediated Immune Responses to *Listeria* Infection by Repeated Exposure to Diesel Exhaust Particles in Brown Norway Rats. *Toxicol. Sci.* **77**, 263–271.
- Yin, X. J., Schafer, R., Ma, J. Y., Antonini, J. M., Weissman, D. D., Siegel, P. D., Barger, M. W., Roberts, J. R., and Ma, J. K. (2002). Alteration of pulmonary immunity to *Listeria monocytogenes* by diesel exhaust particles (DEPs). I. Effects of DEPs on early pulmonary responses. *Environ. Health Perspect.* **110**, 1105–1111.
- Zhu, S., Haddad, I. Y., and Matalon, S. (1996). Nitration of surfactant protein A (SP-A) tyrosine residues results in decreased mannose binding ability. *Arch. Biochem. Biophys.* **333**, 282–290.
- Zhu, S., Kachel, D. L., Martin, W. J., II, and Matalon, S. (1998). Nitrated SP-A does not enhance adherence of *Pneumocystis carinii* to alveolar macrophages. *Am. J. Physiol.* **275**, L1031–L1039.