Effect of Preexposure to Ultrafine Carbon Black on Respiratory Syncytial Virus Infection in Mice

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Epidemiological studies have indicated that exposure to elevated levels of particulate matter exacerbates several pulmonary diseases, including asthma, bronchitis, and viral infections. Respiratory syncytial virus (RSV) is the major cause of bronchiolitis and pneumonia in infants and may lead to the development of asthma in childhood. To determine whether particle exposure modulates the immune response to RSV, eight-week-old female BALB/c mice received an intratracheal (i.t.) instillation of either 40 μg ultrafine carbon black (CB) particles or vehicle. The following day, mice were i.t. instilled with either 109 pfu RSV or uninfected media. End points were examined 1, 2, 4, 7, and 10 days after RSV infection. Compared with RSV alone, tumor necrosis factor-α (TNF-α) protein was reduced in the bronchoalveolar lavage fluid (BALF) on days 1 and 2 of infection; there was also a reduction in BALF lymphocyte numbers on day 4, which correlated with reductions in both IFN-γ-inducible protein (IP-10), lymphotactin, and IFN-γ mRNAs in the lungs of RSV + CB mice. Multiprobe ribonuclease protection assays of RSV + CB lung tissue showed no changes in the RSV-associated chemokines regulated upon activation, normal T cell expressed and secreted (RANTES), eotaxin, monocyte chemoattractant protein (MCP-1), macrophage inflammatory protein (MIP)-1α or MIP-1β. Viral titers in RSV + CB mice were lower than RSV on days 2–4 of infection. By day 7 of infection, however, neutrophil numbers, proinflammatory cytokine mRNA expression, and protein levels of TNF-α and the Th2 cytokine interleukin (IL)-13 were increased in the lungs of RSV + CB mice, indicating an exacerbation of infection. These data indicate that preexposure to ultrafine particles induces an inflammatory milieu promoting allergic immune responses rather than IFNγ production necessary for microbial defense.

Key Words: RSV; chemokine; cytokine; ultrafine particles; alveolar macrophages; lymphocytes; mouse; ribonuclease protection assay.

Recent epidemiological studies have shown correlations between elevated levels of particulate matter (PM) and increased incidence of pulmonary infections and airway hyperreactivity (Pope and Dockery, 1992; Thurston et al., 1994). Morbidity or mortality due to PM exposure correlated with exacerbations of chronic obstructive pulmonary disease (COPD), asthma, or viral infections (Bascom et al., 1996; Pope et al., 1992; Thurston et al., 1992). Studies have revealed increases in upper respiratory symptoms such as rhinitis, sinusitis, sore throat, wet cough, and head cold and in lower respiratory symptoms such as wheezing, shortness of breath, and chest pain following episodes of elevated ambient PM (Dockery and Pope, 1994).

Recently, ultrafine particles (aerodynamic diameter <0.1 μm) have been implicated in the exacerbation of respiratory diseases (Peters et al., 1997). Particles in the ultraxine size range may affect a greater epithelial surface area in the lung and are thus more likely to deposit in the lower airways and the alveolar region for phagocytosis by alveolar macrophages (Öberdörster et al., 1994).

While the effects of PM on animal models of asthma (Gavett et al., 1999; Lambert et al., 1999, 2000) have been studied, there is a paucity of research on the effects of PM on animal models of human viral infections. Respiratory syncytial virus (RSV) is an important etiological agent of acute respiratory tract infections in children (Simoes, 1999). Elderly and otherwise immunocompromised individuals are also prone to infection with this virus (Mlinaric-Galinovic et al., 1996). RSV is a negative-sense enveloped RNA virus that is classified as a pneumovirus within the paramyxovirus family. The virus first replicates in the epithelial cells of the upper respiratory tract and then migrates to the bronchoalveolar region where it induces inflammation in terminal bronchioles and alveoli; epithelial cell necrosis and mucus plugs lead to airway obstruction and hyperinflation of the alveoli (Shay et al., 1999; Simoes, 1999). Severe RSV is strongly associated with wheezing, childhood asthma, and repeated episodes of bronchospastic bronchitis, which can continue into adulthood (Kimpen, 2000; Simoes, 2001).

Despite the lack of in vivo studies, cell culture systems have been used to address the effects of PM on RSV infection. Becker and Soukup (1999) demonstrated that the macrophage inflammatory response to RSV and viral uptake were attenuated in the presence of PM, suggesting a mechanism for increased spread of infection and viral pneumonia in PM-exposed populations.

The purpose of the present study was to develop a mouse model of human RSV infection in which ultrafine particles could be studied to determine their effects on RSV infection. This model could be used to assess the effects of PM exposure on RSV infection and to screen chemical agents that may attenuate the immune response to RSV and viral uptake in the presence of PM.
model of RSV infection to examine the effects of ultrafine carbon black particles on the pulmonary immune response to the virus. While chemically inert, carbon black particles have inflammmogenic properties in experimental models (Li et al., 1999) and are environmentally relevant because carbonaceous material comprises approximately 50% of the mass of PM and forms the core of many ambient air particles, such as diesel exhaust (Clarke et al., 1984). Our hypothesis is that preexposure to ultrafine particles might alter viral immune responses via the attenuation of macrophage function or damage to the airway epithelium, resulting in exacerbation of both RSV-induced pulmonary inflammation and lung function parameters. Our objectives were to examine the time course of responses during RSV infection with or without particle exposure and to determine the mediators responsible for physiologic responses. We show here that RSV infection in mice mimics human viral infection by increasing lymphocyte and neutrophil recruitment to the airways and upregulating proinflammatory cytokines and chemokines in the lung. Preexposure to ultrafine CB modulated some, but not all, of these RSV-induced responses.

MATERIALS AND METHODS

Animals. Pathogen-free, 6-week-old female BALB/c mice were obtained from Charles River Laboratories (Raleigh, NC), fed ad libitum, and housed in polycarbonate cages in an AAALAC-accredited animal facility that was humidity- and temperature-controlled and HEPA-filtered. Mice used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the CIIT Centers for Health Research (CIIT).

Carbon black and RSV. Ultrafine carbon black (surface area = 150 m²/g) was obtained from Columbian Chemicals Company (Marietta, GA). The particles were deemed free of endotoxin by a Limulus amebocyte lysate agglutination assay (BioWhittaker, Walkersville, MD). Direct-pelleted respiratory syncytial virus (RSV, A-2 strain) was obtained from Advanced Biotechnologies Incorporated (Columbia, MD) and had a stock concentration of 5 × 10⁵ pfus/ml. RSV was kept at −70°C in minimum essential medium with Earle’s salts plus 10% FBS and 50 μg/ml gentamicin until use.

Particle and virus exposures. Preliminary dose-response studies with 20, 40, and 80 μg carbon black were conducted to determine the dose required to produce neutrophilic inflammation at 24 h post instillation. The midrange dose of 40 μg was chosen because it induced a moderate neutrophilic inflammatory response (data not shown). Thus, at eight weeks of age, mice (18–20 g) were anesthetized by isoflurane inhalation and received intratracheal (i.t.) instillations of either 40 μg carbon black in 100 μl sterile saline (carbon black solutions were sonicated for 5 min prior to use), or 100 μl sterile saline using a 1-ml syringe and round-tip needle. One day later, mice were infected by i.t. instillation of 200 μl medium containing 1 × 10⁵ pfus RSV, or were sham-infected with 200 μl of uninfected medium. The experiment was repeated twice; end points were assessed 1, 2, 4, 7, and 14 days (experiment 1) or 1, 2, 4, 7, and 10 days (experiment 2) following RSV or sham instillations, for a total of 10 mice per group on days 2, 4, and 7, and 5 mice per group on days 1 and 10. To verify infection in the mice, lung homogenates were subject to an ELISA assay using antibodies to RSV (ViroStat, Portland, ME). This ELISA has been shown to correlate well with the standard plaque assays typically used to determine viral titer in lung tissue (Malley et al., 2000).

Bronchoalveolar lavage and cell differentials. Mice were euthanized by anesthesia with 50 mg/kg Nembutal (Abbott Laboratories, Chicago, IL) and lavaged with two separate 1-ml aliquots of PBS. Approximately 1.8 ml of the 2.0-ml total volume (90%) was consistently recovered. Supernatants were collected after centrifugation (300 × g, 10 min) and stored at −80°C for cytokine protein analysis. Cell pellets were resuspended in F12 media ( Gibco, Gaithersburg, MD) and counted (Coulter, Hialeah, FL). Slides were made (Cytospin 3, Shandon, Pittsburgh, PA) and stained with Diff Quik (American Scientific Inc., Sewickly, PA). Approximately 200 cells were differentiated per slide. For assessment of pulmonary inflammation, left lung lobes from day 7 were removed and inflated with Tissue-Tek OCT (Sakura Finetek U.S.A., Torrence, CA), placed on a brass chuck and embedded in OCT, snap-frozen in liquid nitrogen, and stored at −80°C until sectioned. Lungs from four per treatment group were sectioned at 5 μm on a cryostat, and the tissues were placed on glass slides and stained with hematoxylin and eosin for pathology. Because of the observed spike in neutrophil numbers on day 7, we exposed mice in a separate experiment to CB prior to RSV instillation exactly as described previously and extracted whole, unlavaged lungs on day 7 for further pathological analyses. Briefly, lungs (n = 3 per treatment group) were perfused and fixed with 10% buffered formalin. Tissues were embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. All tissues were analyzed by a pathologist for the degree of pulmonary inflammation due to RSV infection, and the effect of carbon black particles on viral-induced inflammation.

Cytokine protein quantitation. Whole lungs were homogenized in PBS supplemented with protease inhibitors (Roche Diagnostics, Manheim, Germany), and were centrifuged (500 × g, 30 min). BAL and homogenate supernatants were analyzed using commercial enzyme-linked immunosorbent assay (ELISA) kits for TNF-α (BALF supernatants; Biosource International, Camarillo, CA) and IL-13 (lung homogenates; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Quantitation of interferon-gamma inducible protein 10 (IP-10) mRNA. Right lungs were snap-frozen in liquid nitrogen and stored at −80°C until total RNA was isolated by the diethylpyrocarbon phenol chloroform method using RNA STAT-60 (Tel-Test, Friendswood, TX). A gene-specific relative RT-PCR kit (Ambion, Austin, TX) was used for IP-10 quantitation. Briefly, total RNA (2.5 μg) was reverse transcribed at 42°C for 1 h with 2.5 mM dNTPs, 50 μM random primers, 10× RT-PCR buffer, 100 units M-MuLV, and RNase inhibitor. Specific cDNA for IP-10 was amplified by adding 5 μl RT reaction to 27.5 μl 10 × PCR buffer, 2.5 mM dNTPs, 5 μM primers, 1.4 μl DNA polymerase, 4 μl 18S PCR primer pair, and 6 μl 18S competimers and heated to 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s for 35 cycles. PCR products were run on a 2% agarose gel containing ethidium bromide and visualized under UV illumination. Band intensities of IP-10 were normalized to those of the 18s rRNA subunit using Alpha Ease densitometry software (Alpha Innotech, San Leandro, CA).

Ribonucleic protection assays. Chemokine mRNA expression was determined by ribonucleic protection assay (RPA) using the RiboQuant kit (BD-PharMingen, San Diego, CA) according to the manufacturer’s protocol. The multiprobe template mCK-5 contains DNA templates for lymphotactin, RANTES, eotaxin, MIP-1β, MIP-1α, MIP-2, MCP-1, and TCA-3 and housekeeping genes L32 and GAPDH. Other probes used in these experiments included RNA templates for interleukins 10, 13, 15, 9, 2, 6, and IFN-γ (mCK-1) and interleukins 10, 1α, 1β, 1Rα, 6, and IFN-γ (mCK-2) plus L32 and GAPDH genes. Briefly, after labeling the probe with 32P dUTP and hybridizing with 10 μg total RNA sample, were loaded onto a 6% Urea PAGE gel (Invitrogen) and identified by comparing migration distances to the unprotected probe. Band intensities were determined using ImageQuant software on a Phosphorimager SI (both by Molecular Dynamics, Sunnyvale, CA). Samples were normalized to their respective housekeeping gene, L32.

Statistical analyses. Within each experiment, treatment groups were compared by ANOVA using statistical packages from SAS Institute, Inc. (Cary, NC), or Statview (Abacus Concepts, Berkeley, CA). If the F-test was significant, Tukey’s test was used to find differences among treatments. Bonferroni correction for multiple subtesting was also used throughout the statistical analyses. A p value < 0.05 was considered significant.
RESULTS

Effect of Ultrafine Particle Exposure on RSV-Induced Pulmonary Inflammation

Macrophage numbers were similar among the four treatment groups except on day 4, when macrophage numbers of CB-exposed mice exceeded controls and on days 7 and 10, when both RSV-exposed groups exceeded controls (Fig. 1). Neutrophil numbers were elevated in all treatment groups compared to controls 24 h postinfection (48 h post-CB), and continued to be significantly higher than controls in both RSV-infected groups until day 10 postinfection, when the inflammation subsided. An increase in neutrophil numbers was evident in the RSV + CB group on day 7, and was observed in two separate experiments (n = 10). Both RSV-infected groups had significant elevations in BAL lymphocyte numbers compared to CB and saline controls throughout the time course. By 48 h postinfection, however, a trend appeared for a reduction in lymphocyte numbers in the RSV + CB group compared with RSV alone that reached statistical significance on day 4 of infection. Lymphocyte numbers increased dramatically on days 7 and 10 but were not different between RSV-infected groups. Eosinophils were negligible in this model. Viral clearance in the lung was unaffected by CB exposure (data not shown).

Effect of Ultrafine Particles, RSV, or Both on TNF-α and IL-13 Protein Levels in the Lung

TNF-α protein was significantly elevated in the BALF of RSV-infected mice compared to CB and saline controls on days 1 and 2, but RSV + CB mice had significantly lower TNF-α levels compared to RSV alone (Fig. 2A). On days 4 and 7 of infection, RSV + CB TNF levels were higher than RSV alone. By day 10, TNF had returned to control levels. IL-13 proteins in lung homogenates of RSV + CB mice were significantly elevated compared to control on days 4, 7, and 10 (Fig. 2B). On day 7 of infection, RSV + CB mice had nearly three-fold higher IL-13 levels than RSV alone; this result was observed in both sets of experiments (n = 10).

FIG. 1. Inflammatory cell numbers in BAL fluid of mice 1, 2, 4, 7, and 10 days following infection with RSV. Values shown are means ± SE (n = 5–10 mice per group). *Different from control; groups sharing the same letter are not significantly different from one another by ANOVA, p < 0.05.

FIG. 2. (A) TNF-α protein levels in BALF. (B) IL-13 protein levels in lung homogenates measured by ELISA on days 1, 2, 4, 7, and 10 during infection. Values are mean pg protein per ml ± SE (n = 5–10 mice per group). *Different from control; †different from RSV by ANOVA, p < 0.05.
Effect of Ultrafine CB on RSV-Induced Interferon Inducible Protein (IP-10) mRNA in the Lung

Messenger RNA for IP-10, an early response chemokine produced by bronchial epithelial cells, macrophages, and neutrophils that induces a Th1 phenotype, was detectable on day 1 of infection (Fig. 3). Compared to CB and control groups, RSV-infected animals had a significant elevation in mRNA expression of IP-10. RSV + CB mice had significantly reduced IP-10 mRNA expression compared with the RSV group and was not different from control. CB-exposed mice had significantly reduced IP-10 mRNA expression compared to all groups.

Effect of Ultrafine CB and/or RSV on Chemokine and Cytokine mRNAs by RPA

On day 1 postinfection (the peak of mRNA expression during the time course), IL-15, IL-2, IL-6, and IFN-γ mRNAs were elevated in the lung tissue of RSV-infected mice compared to controls (Fig. 4A). IFN-γ mRNA was significantly reduced in RSV + CB mice compared to mice exposed only to RSV. Similar reductions in IFN-γ mRNA expression were found on day 4 of infection (data not shown), which correlates with the significant reduction in lymphocytes on day 4 shown in Figure 1. IL-1α, IL-1β, IL-1Ra (IL-1 receptor antagonist), and IL-6, mRNAs were elevated due to RSV infection (Fig. 4A), but IL-1Ra was significantly reduced in RSV + CB mice compared to RSV alone. All chemokine mRNAs measured in Figure 4A were elevated due to RSV infection except lypho-tactin (Ltn), which was significantly reduced in RSV + CB mice compared to RSV alone. On day 7 of infection (Fig. 4B), IFN-γ mRNA was increased in RSV + CB compared with RSV alone, as were proinflammatory cytokine mRNAs IL-6, IL-1β, and IL-1 receptor antagonist (IL-1Ra) and the T-cell proliferation-inducing cytokine, IL-2. IL-13 mRNA was elevated in both RSV infected groups on day 7, but unlike protein expression data, no effects of CB exposure on IL-13 mRNA expression were noted.

Histopathological Effects of RSV with or without CB Exposure

At day 7, CB + RSV mice differed from RSV alone in having a more severe pulmonary inflammation. Inflammation in both groups of animals was primarily angiocentric characterized by lymphoid and mononuclear cells cuffing arterioles and venules. Similar inflammatory cell aggregates were noted in peribronchiolar regions. While the peribronchiolar and perivascular cuffing was similar in composition in both groups, it was more severe in the CB + RSV animals (Fig. 5). Interstitial inflammation in alveolar parenchymal regions, noted as septal thickening, was limited to CB + RSV mice. In addition, several CB + RSV mice had regions of lung characterized by bronchiolar exudate with numerous degenerate neutrophils and occasional basophilic bacterial colonies indicative of a bacterial bronchopneumonia (Figs. 5C and 5D). The fact that only CB + RSV exposure, and not CB exposure alone, led to bacterial secondary pneumonias, suggests that coexposure rather than a bacterial contamination of particles, is responsible for the pulmonary changes.

DISCUSSION

In this study, we show for the first time in an in vivo model an interactive effect between a prototypic ultrafine ambient air
particle, carbon black (CB), and an important human respiratory virus, RSV. CB particles were used in this study because of our interest in the effects of particle size and preexisting neutrophilic inflammation on RSV infection. We did not want to confound our studies with frank toxic effects such as those seen with diesel exhaust and fly ash particles.

CB pretreatment tipped the immune response away from a Th1 phenotype and toward Th2, as evidenced by an increase in the Th2 cytokine, IL-13, and decreases in TNF-α and the Th1 cytokines IP-10 and IFNγ in CB-exposed RSV-infected mice. Other investigators have observed similar Th2-enhancing effects of CB preexposure in models of ovalbumin sensitization (Lovik et al., 1997; van Zijverden et al., 2000). In accordance with other studies (Li et al., 1999) exposure of BALB/c mice to ultrafine CB induced a significant, acute neutrophilic pulmonary inflammation. Because neutrophils are capable of generating reactive oxygen metabolites that may damage airway epithelium (Wang and Forsyth, 2000; Wang et al., 1998), we initially hypothesized that exposure to ultrafine CB particles prior to RSV infection would allow for enhanced viral access and replication in the epithelium. No differences in viral replication or clearance were observed in our studies, however (data not shown).

Resistance to viral infection requires cooperation between macrophages (innate immune response) and T cells (acquired immune response), as well as certain inflammatory cytokines

**FIG. 4.** Cytokine and chemokine mRNA expression in lung tissue of RSV-infected mice with or without CB pretreatment on day 1 (A) and day 7 (B) of RSV infection. Expression of 17 murine cytokine mRNAs was investigated by RPA. The quantity of each mRNA was determined by the band intensity using densitometry. Values shown are mean densities ± SE (n = 5) relative to that of L32. *Different from uninfected controls; groups sharing the same letter are not significantly different by ANOVA, p < 0.05.
secreted by each cell type. TNF-α, produced by activated macrophages, is an early response cytokine important for its ability to signal cells to generate additional cytokines necessary to maintain the immune response (Kunkel et al., 1999, Luster et al., 1999). TNF-α has antiviral activity (Mestan et al., 1986), is a chemoattractant for T lymphocytes, and may play a role in recruiting cells for specific RSV immune responses (Franke-Ullmann et al., 1995). We show here that CB exposure attenuates RSV-induced TNF-α production in the lung, which suggests a blunting of the innate immune response, suppression of macrophage function, and subsequent reduction of immune signaling to T cells.

Another macrophage-derived inflammatory mediator, IP-10, is a CXC chemokine that plays an important role in Th1 immunity. It is a potent activator and chemoattractant for neutrophils that interact with the CXCR3 receptor expressed on Th1 cells (Neville et al., 1997). IP-10 is also a chemoattractant for T cells, monocytes, and NK cells and participates in the innate immune response by the initiation of a Th1 polarized adaptive immune response to viral infection (Wiley et al., 2001). In our study, IP-10 mRNA was enhanced in the lung 24 h post-RSV exposure but was significantly reduced in CB + RSV-exposed mice. Additionally, CB-exposed mice had reductions in IP-10 mRNA compared to saline-exposed controls, suggesting a shift toward a Th2 phenotype due to particle exposure alone. Our findings of more severe lung pathology (secondary bacterial infection) and increased IL-13 in CB + RSV mice on day 7 of infection support the notion that the reduction of this Th1-inducing chemokine may have promoted the exacerbation of RSV infection.
The kinetics of the recruitment of RSV-induced immune cells to the lung appeared to be altered in CB-exposed mice such that later time points were adversely affected. For example, lymphocyte numbers steadily increased throughout the time course of RSV infection, but lagged behind in CB + RSV-exposed mice, and were statistically lower on day 4 of infection. This decrease in lymphocyte numbers, presumably CD4+ T-helper 1 and CD8+ cytotoxic T cell populations resulted in reductions in both IFN-γ and lymphotactin mRNAs in the lung tissue on day 1 and on day 4 (data not shown). Attenuation of these Th1 T lymphocyte-derived factors could compromise an individual with respect to immune defense against pulmonary viruses (Borthwick et al., 1997; Giancarlo et al., 1996; Müller et al., 1995), and may have promoted RSV disease progression. Thus, by day 7 of infection, lymphocyte numbers in CB + RSV-exposed mice increased and were comparable to those of mice infected with RSV alone. There was also a trend for an increase in neutrophil numbers in CB + RSV mice on day 7, suggesting an exacerbation of infectious disease. Pulmonary inflammation in CB + RSV mice on day 7 of our study was accompanied by increased TNF-α protein in the BALF, and elevations in mRNA expression of the cytokines IL-6 (proinflammatory), IFN-γ (Th1), IL-1 receptor antagonist (IL-1Ra), and IL-2 (T cell proliferative factor). IL-1Ra production is triggered by LPS (Janson et al., 1991) as well as viral gene products (Kline et al., 1994).

In summary, our studies show that preexposure of BALB/c mice to ultrafine particles did not enhance RSV replication, nor significantly affect viral clearance. However, a Th2 environment may have been created in the lung by the CB-induced inflammatory milieu, promoting allergic immune responses rather than Th1 responses necessary for microbial defense.

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REFERENCES


Oberdörster, G., Ferin, J., and Lehnert, B. E. (1994). Correlation between...