Ultrafine Carbon Black Particles Enhance Respiratory Syncytial Virus-Induced Airway Reactivity, Pulmonary Inflammation, and Chemokine Expression

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Exposure to particulate matter (PM) may exacerbate preexisting respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), bronchitis, and pneumonia. However, few experimental studies have addressed the effects of PM on lower respiratory tract (LRT) viral infection. Respiratory syncytial virus (RSV) is a major etiological agent for LRT infections in infants, the elderly, and the immunocompromised and may lead to chronic wheezing and the development of asthma in children. In this study, we examined the effects of carbon black (CB) on RSV-induced pulmonary inflammation, chemokine and cytokine expression, and airway hyperresponsiveness in a mouse model of RSV. Female BALB/c mice were instilled via the trachea (i.t.) with 1 × 10⁴ plaque forming units (pfu) RSV or with uninfected culture media. On day 3 of infection, mice were i.t. instilled with either 40 µg ultrafine CB particles or with saline. End points were examined on days 4, 5, 7, and 14 of RSV infection. Viral titer and clearance in the lung were unaffected by CB exposure. Neutrophil numbers were elevated on days 4 and 7, and lymphocyte numbers were higher on days 4 and 14 of infection in CB-exposed, RSV-infected mice. CB exposure also enhanced RSV-induced airway hyperresponsiveness to methacholine, bronchoalveolar lavage (BAL) total protein, and virus-associated chemokines monocyte chemoattractant protein (MCP-1), macrophage inflammatory protein (MIP-1α), and regulated upon activation, normal T cell expressed and secreted (RANTES). MIP-1α mRNA expression was increased in the alveolar epithelium, where ultrafine particles deposit in the lung. These data demonstrate a synergistic effect of ultrafine CB particles on RSV infection, and suggest a potential mechanism for increased respiratory infections in human populations after PM exposure.

Key Words: RSV; chemokine; cytokine; ultrafine particles; airway hyperresponsiveness; laser capture microdissection; mouse.

This RNA virus in the paramyxovirus family also affects immunocompromised individuals such as the elderly, often leading to pneumonia, bronchitis, or death (Mlinaric-Galinovic et al., 1996). Genetically susceptible individuals may develop an inappropriate Th2 immune response to the viral infection, promoting the exacerbation of viral infections and the development of persistent wheezing and asthma later in childhood (Kimpen, 2001).

Particulate matter air pollutants have been shown to exacerbate a variety of pulmonary disorders, including chronic obstructive pulmonary disease (COPD; Schwartz, 1994; Sunyer and Basagana, 2001), asthma (Lipsett et al., 1997; Peters et al., 1997a), and lower respiratory tract infections, especially in children and the elderly (Braun-Fahrlander et al., 1992; Hwang and Chan, 2002). Specifically, particles less than 0.1 µm in mass median aerodynamic diameter (MMAD; a density-dependent unit of measure used to describe the diameter of the particle) have been implicated in these adverse health effects (Peters et al., 1997b; Utell and Frampton, 2000).

Ultrafine particles are deemed more toxic because they can penetrate to the alveolar regions in the deep lung and may thus be systemically distributed in the bloodstream (Seaton et al., 1995). Ultrafine particles may also affect a greater surface area in the lung, causing more inflammation than larger particles (Li et al., 1999; Ober dorster et al., 1994; Tran et al., 2000).

We used carbon black in our studies because it is a useful prototypical particle for the study of the mechanisms of PM effects. Since it is relatively inert, the effects of particle size can be examined without confounding factors such as toxicity. Carbon black is also relevant to human exposures because carbonaceous material comprises approximately 50% of the mass of PM and forms the core of many particles (Clarke et al., 1984).

The mechanisms for PM-induced health effects found in epidemiological studies have been studied in animal models of asthma (Lambert et al., 1999, 2000) and bronchitis (Kodavanti and Costa, 2001), but few studies have addressed PM effects in animal models of human viral infections such as RSV. BALB/c mice have typically been used in models of RSV infection with respect to vaccine development (Graham et al., 1988). Herein,
we describe a BALB/c mouse model of RSV infection developed to understand the mechanisms by which ultrafine particulate matter exacerbates viral infection. Our model mimics many of the features of RSV infection in humans, including neutrophilic pulmonary inflammation, airway hyperresponsiveness to cholinergics, upregulation of CC chemokines such as RANTES, MCP-1, and MIP-1α, and viral replication in alveolar epithelium. We have previously shown that preexposure to ultrafine CB promotes a Th2 environment in the lung, leading to the development of secondary bronchopneumonia later in the time course of infection (Lambert et al., 2003). In the present study, we sought to determine the effects of ultrafine CB exposure during RSV infection by examining airway hyperresponsiveness to methacholine, pulmonary injury and inflammation and chemokine expression, and viral titer in the lung.

MATERIALS AND METHODS

Animals. Pathogen-free, six-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 CHT Centers for Health Research.

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 amoebocyte lysate agglutination assay (BioWhittaker, Walkersville, MD). Stock solutions of the particles were sonicated frequently during dosing. 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 pfu/ml. RSV was kept at −80°C in minimum essential media with Earle’s salts plus 10% FBS and 50 μl/ml gentamicin until use. The RSV preparation was thawed, vortexed, and used as received from the vendor.

Particle and virus exposures. At eight weeks of age, mice (18–20 g) were anesthetized by isoflurane inhalation and infected by intratracheal (i.t.) instillation. One mouse was infused with each of the treatments, with 50 μl of the respective solution instilled into the lungs using a 33-gauge needle. One hundred μl of saline was instilled into the contralateral lung to serve as an isovolume control. Two days later, mice were euthanized 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 total protein measurements (an indicator of lung permeability). Protein concentration was measured following the method of Bradford (1976). The cell pellets from both lavages were combined and 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. Left lung lobes were removed at each time point and inflated with Tissue-Tek OCT (Sakura Finetek U.S.A., Torrance, CA), placed on a brass chuck and embedded in OCT, snap-frozen in liquid nitrogen, and stored at −80°C until sectioned for laser capture microdissection.

Chemokine protein quantitation. Right lung lobes were homogenized in PBS supplemented with protease inhibitors (Roche Diagnostics, Manheim, Germany), and centrifuged (500 × g, 30 min). Lung homogenate supernatants were stored at −80°C until analyzed using commercial enzyme-linked immunosorbant assay (ELISA) kits for MIP-1α, MCP-1, and RANTES (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

RSV antigen quantitation. Lung homogenates were also subjected to an ELISA assay to determine relative levels of RSV antigen in lung tissue. This ELISA has been shown to correlate well with the standard plaque assays typically used to determine viral titer in lung tissue but has advantages over plaque assays because it measures both infectious and noninfectious viral particles, both of which may elicit an inflammatory response (Malty et al., 2000). For the assay, 96-well plates were coated overnight at 4°C with 100 μl polyclonal goat anti-RSV (diluted 1/500, ViroStat, Portland, ME), washed with PBS containing 0.05% Tween-20 (Sigma), blocked at room temperature (RT) for 1 h with 200 μl PBS-0.05% Tween-20 containing 1% bovine serum albumin (Sigma), and washed again. Lung homogenates (diluted 1:5) were applied in duplicate and incubated overnight at 4°C. After washing, biotinylated goat anti-RSV (ViroStat) was added and incubated for 1 h RT and washed; plates were incubated for 1 h RT with horseradish peroxidase streptavidin (Zymed), washed again, and incubated for 15 min RT with TM Blue (Dako). Plates were read at 650 nm with a Thermomax ELISA plate reader (Molecular Devices).

Laser capture microdissection of alveolar epithelium. Lungs from five mice per treatment group (on day 4 of infection) were sectioned at 5 μm on a cryostat. The frozen tissues were placed on clean glass slides (Fisher Scientific, Pittsburgh, PA) and were immediately fixed in 95% ethanol for 1 min followed by ribonuclease (RNase)-free water (15 s), Mayer’s hematoxylin (15 s), 0.5% lithium carbonate (30 s) RNase free water (30 s), 70% ethanol (30 s), 80% ethanol (30 s), 100% ethanol (30 s), then dehydrated in graded ethanol (30 s each), and cleaned in xylene (1 min). Slides were air-dried and kept dessicated to prevent the activation of RNases. LCM of alveolar epithelial cells was performed using a PixCell II System (Arcturus Engineering, Mountain View, CA) with the following settings: laser diameter, 7 μm; pulse duration, 1.5 ms; and amplitude, 45 mW. The laser beam activates a transfer film (cap) which bonds specifically to cells targeted by microscopy within the tissue section, and when the cap is lifted off of the tissue section, the selected cells (in this case alveolar epithelial cells) are contained within the cap, leaving behind all unwanted cells. Cells were extracted from the caps using cell lysis buffer containing β-mercaptoethanol and pooled among treatment groups for a total of four samples.

Reverse transcription. Total RNA was isolated from cell lysates using a StrataPrep® Total RNA Microprep Kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Genomic DNA was removed from all RNA samples by DNase I treatment. The quantity of 30 μl of RNA extract from pooled laser-captured cells containing unknown amounts of RNA was reverse-transcribed using random hexamers (Applied Biosystems [ABI], Foster City, CA) and MuLV RT (ABI) in 100 μl total volume at 25°C for 10 min, 48°C for 30 min, and 94°C for 5 min. The resulting complementary DNA (cDNA) was used as template in the subsequent real-time PCR reaction. Primers for MIP-1α and the housekeeping gene L32 were designed using Primer Express™ software (Perkin Elmer, Foster City, CA; Table 1).
Real-time PCR. Quantitative RT-PCR was performed using SYBR® Green (ABI) with the ABI Prism® 7700 Sequence Detection System (ABI). Primer specificity was confirmed by ethidium bromide staining of the predicted size of the PCR products on an agarose gel. Primer efficiencies, reaction conditions, and data analysis were performed using a PCR reagent kit (ABI) according to the manufacturer’s protocol. Briefly, 2 μl cDNA was added to a master mixture containing all reagents necessary for PCR, including SYBR Green, 100 nM primers, and AmpliTaq Gold enzyme. All samples were run in triplicate using L32 as the calibrator gene since L32 levels did not change significantly from RSV or CB exposure (data not shown).

To determine the effect of CB + RSV exposure on the expression of MIP-1α, the threshold cycle (Ct) values for the triplicate reactions were averaged, and the average L32 Ct value for each sample was subtracted from the average Ct value for MIP-1α to obtain a normalized Ct value. The normalized Ct values for the control animals were subtracted from the normalized Ct value for each treated sample to obtain a relative Ct value. To obtain the relative expression of a particular gene as a result of RSV or CB exposure, the following function was used: fold change = 2−[(Ct MIP-1α)−(Ct L32)]. In addition, these fold changes were adjusted to reflect the amplification efficiencies of the primer sets (Pfaffl, 2001). All expression levels reported were expressed relative to those determined in vehicle-exposed animals.

Statistical analyses. Within each experiment, treatment groups were compared by ANOVA using Statview (Abacus Concepts, Berkeley, CA). Pairwise comparisons following an overall significant ANOVA were performed using Fisher’s protected least significant test or Scheffe’s F test. A p value < 0.05 was considered significant.

RESULTS

Effect of Ultrafine CB on RSV-Induced Inflammation

BAL cell differentials were evaluated for numbers of macrophages, lymphocytes, and neutrophils (eosinophils were not significant in this model) on days 4, 5, 7, and 14 of infection (days 1, 2, 4, and 11 post-CB instillation). On day 4 of infection (one day post-CB), macrophage numbers were significantly elevated in CB-exposed mice compared to all treatment groups (Fig. 1). Lymphocyte numbers were enhanced due to RSV infection but were significantly greater in CB-exposed RSV infected mice on days 4 and 14 of infection. CB exposure induced an acute neutrophilic response on day 4 (one day post-CB) that quickly subsided by day 5 (two days post-CB), and promoted enhanced numbers in RSV + CB mice compared with RSV alone on day 4. Neutrophil numbers were also greater in RSV + CB mice on day 7 of infection compared with RSV alone.

Effect of Ultrafine CB Exposure on Lung Permeability

Total protein levels, a measure of epithelial cell integrity and lung injury, were significantly enhanced in the BAL of CB + RSV mice over RSV alone and vehicle controls on day 4 of infection (one day post-CB) only (Fig. 2). On days 5 and 7 of infection, both RSV-infected groups had significant increases in total protein compared to CB or vehicle control. On day 14, lung injury was still apparent in RSV + CB mice but had returned to control levels in all other treatment groups.

Effect of CB Exposure on RSV Antigen Titers in the Lung

There was no apparent effect of CB exposure on RSV antigen titer or rate of viral clearance (Fig. 3). In both RSV-treated groups, RSV titers were at their maximal levels on days 4 and 5 of infection, and the virus was rapidly cleared by day 7. RSV levels were indistinguishable from uninfected animals on day 14.

Effect of CB Exposure on RSV-Induced Airway Hyperresponsiveness

Airway hyperresponsiveness measurements were recorded on days 4, 5, 7, and 14 of infection (Fig. 4). On day 4 (one day after CB exposure), both CB-exposed groups of mice were hyperresponsive to methacholine compared to RSV alone and saline vehicle. On day 5 of infection, RSV-only exposed mice and CB-only exposed mice were hyperresponsive compared to controls, but RSV + CB-exposed mice had significantly elevated enhanced pause values compared to all other groups. No significant differences were found on days 7 and 14 of infection.

Effect of CB Exposure on RSV-Induced Chemokine Proteins in the Lung

RANTES protein levels were elevated throughout the time course in RSV-infected mice regardless of CB treatment (Fig. 5), but RANTES remained significantly elevated in RSV + CB-treated mice on day 14 compared to all groups. A synergistic effect of virus and particle exposure was evident by a significant elevation of MIP-1α and MCP-1 proteins in RSV + CB-exposed mice on day 4 of infection (Fig. 5). This coincided

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with a significant elevation in lung permeability in RSV + CB mice shown in Figure 2. MIP-1α and MCP-1 proteins remained elevated in both RSV-infected groups on days 5 and 7 of infection, but MCP-1 returned to control levels in all groups on day 14. MIP-1α remained elevated only in RSV + CB mice on day 14.

**Effect of CB Exposure on MIP-1α mRNA Expression in Alveolar Epithelium**

We used real-time PCR to analyze cDNA from laser-captured alveolar epithelial cells on day 4 of infection. After normalization to the calibrator gene (L32), a 40-fold increase in MIP-1α mRNA expression was found in RSV + CB mice versus vehicle control and RSV-treated mice (Fig. 6). This elevation in mRNA expression was consistent with elevations in protein levels on day 4 (Fig. 5) and indicative of a synergistic effect between particle and viral exposures. CB-exposed mice had a five-fold increase in MIP-1α mRNA over RSV and vehicle controls.

**DISCUSSION**

In the present study, we have demonstrated an exacerbation of RSV-induced airway hyperresponsiveness (AHR), pulmonary inflammation (i.e., increased numbers of neutrophils and lymphocytes), and chemokine expression by exposure to an inert ultrafine particle, carbon black. Viral clearance and replication in the lung were unaffected by CB exposure.

Carbon black exposure by either inhalation or instillation in experimental animals has been shown to induce neutrophilic inflammation, chemokine expression, and even pulmonary tumors in rat chronic exposure scenarios (Driscoll et al., 1996). The mechanism for these effects is thought to be increased production of reactive oxygen species by alveolar macrophages (Li et al., 1999). We show in our model that acute neutrophilia caused by CB instillation exacerbates inflammatory end points in RSV-exposed mice and causes an increase in both lung permeability (as measured by increased BAL total proteins) and RSV-induced chemokines throughout the time course of infection. Our data suggest that production of chemokines by alveolar epithelial cells may induce these deleterious effects.

One of the chemokines synergistically upregulated by RSV and CB coexposure was MIP-1α, a C-C chemokine that is increased in the respiratory secretions of infants with severe RSV bronchiolitis (Garofalo et al., 2001) and is a chemoattractant for neutrophils (Haelens et al., 1996). MIP-1α is preferentially expressed in the lower respiratory tract by alveolar epithelial cells, which is the same cellular location as viral replication in our model (Olszewska-Pazdrak et al., 1998). The importance of MIP-1α as a mediator of RSV-induced inflammatory responses was demonstrated in genetically altered mice with deletions in the MIP-1α gene (−/−); these mice had significant reductions in neutrophilic inflammation compared to wild-type controls (Haeberle et al., 2001). In our model, neutrophil numbers were significantly elevated in CB-exposed mice over RSV- or CB + RSV-exposed mice on day 4 of infection (one day after CB exposure), but MIP-1α protein was
over 3.6-fold and MIP-1α mRNA 30-fold higher in CB + RSV mice compared with CB-exposed mice. This increase in neutrophil numbers in CB-only–exposed mice was probably due to the secretion of another neutrophil chemoattractant, MIP-2 (an IL-8 homologue), which was augmented on day 1 post-CB exposure to (data not shown).

MCP-1 levels were also synergistically enhanced by CB + RSV compared to either CB or RSV treatment alone. Another C-C chemokine, MCP-1 is a chemoattractant for monocytes. The synergistic increase in chemokine expression by coexposure to particles and RSV suggests that the cells secreting these chemokines, alveolar epithelial cells, are in direct contact with particles or the reactive oxygen species generated by the particles. We also observed by immunohistochemical localization that RSV replicated in the alveolar epithelium (unpublished data) precisely where chemokine expression was localized in our model.

As previously stated, day 4 of infection (the peak of RSV infection and 1 day post-CB exposure) was a key time point in our study since lung permeability (as measured by BAL total proteins), neutrophil numbers, and chemokines were synergistically upregulated in RSV + CB mice. This inflammatory event was followed on day 5 by an exacerbation of AHR in RSV + CB-exposed mice compared to RSV or CB alone. It is interesting that both MCP-1 and MIP-1α were upregulated on day 5 in RSV-infected mice regardless of particle exposure but that AHR was only enhanced in RSV + CB mice at this time point. This enhancement of AHR may have been induced by the increased MCP-1 and MIP-1α by RSV + CB exposures the day before, suggesting a time lag in the inducement of AHR by these mediators of pulmonary inflammation or the involvement of some other factor in the enhancement of AHR by CB particles on day 5. MCP-1 neutralization has been shown to alleviate AHR in a mouse model of allergic inflammation, where eosinophils were the primary inflammatory cell, while MIP-1α neutralization only slightly reduced AHR (Gonzalo et al., 1998). RSV infection of neutrophils has been shown to induce degranulation (as measured by myeloperoxidase re-
lease) and MIP-1α production (Jaovisidha et al., 1999). MIP-1α, in turn, activates mast cells and basophils to release histamines (Alam et al., 1992), which may have caused the AHR in our model. Other investigators have reported that the presence of eosinophils and IL-5 are essential for the induction of AHR in RSV-infected mice (Schwarze et al., 1999). In our model, however, RSV induced a neutrophilic, IFN-γ-dominated response. Future studies in this neutrophil-rich, Th1-type inflammation model are needed to address the separate roles of these chemokines in RSV-induced AHR. Another interesting observation that precipitated from our studies is that CB-treated, RSV-infected mice had fewer neutrophils than did mice treated with CB alone, which suggests that RSV infection modulated particle deposition. Since we did not quantify patterns of particle deposition in our model, we can only speculate about this finding. In conclusion, the present study is one of few conducted to examine in vivo particle interactions with an important human virus, RSV. We have shown that ultrafine CB exposure during RSV infection exacerbates pulmonary inflam-

FIG. 4. Airways hyperresponsiveness measurements in mice exposed to methacholine aerosol in a whole-body plethysmograph on days 4, 5, 7, and 14 of infection with RSV (1, 2, 4, and 11 days post-CB). Following a baseline period, enhanced pause (Penh) measurements were averaged over a 20-min exposure to either saline or 80 mg/ml methacholine. Values are mean Penh + SE (n = 5 mice per group). *Significant difference from saline control; †different from RSV by ANOVA, p < 0.05.

FIG. 5. Protein levels of RANTES, MIP-1α, and MCP-1 in lung homogenates by ELISA assay on days 4, 5, 7, and 14 of infection with RSV (1, 2, 4, and 11 days post-CB). Values shown are means + SE (n = 5 mice per group). *Significant difference from saline control; †different from RSV; ‡different from CB by ANOVA, p < 0.05.
mation, lung injury, AHR, and chemokine output. Our data suggest potential interactions between RSV-infected alveolar epithelial cells and ultrafine CB particles.

Future studies should directly address the role of MIP-1\(\alpha\) and MCP-1 in particle-induced exacerbations of pulmonary inflammation and AHR in RSV infection by using transgenic mice lacking these genes or by exogenous administration of monoclonal antibodies in RSV-infected, CB-exposed BALB/c mice.

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