Short-Term Exposure to Aged and Diluted Sidestream Cigarette Smoke Enhances Ozone-Induced Lung Injury in B6C3F1 Mice

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Received April 16, 2001; accepted September 17, 2001

To determine the effects of aged and diluted sidestream cigarette smoke (ADSS) as a surrogate of environmental tobacco smoke (ETS) on ozone-induced lung injury, male B6C3F1 mice were exposed to (1) filtered air (FA), (2) ADSS, (3) ozone, or (4) ADSS followed by ozone (ADSS/ozone). Exposure to ADSS at 30 mg/m³ of total suspended particulates (TSP) for 6 h/day for 3 days, followed by exposure to ozone at 0.5 ppm for 24 h was associated with a significant increase in the number of cells recovered by bronchoalveolar lavage (BAL) compared with exposure to ADSS alone or ozone alone. The proportion of neutrophils and lymphocytes, as well as total protein level in BAL, was also significantly elevated following ADSS/ozone exposure, when compared with all other groups. Within the centriacinar regions of the lungs, the percentage of proliferating cells identified by bromodeoxyuridine (BrdU) labeling was unchanged from control, following exposure to ADSS alone, but was significantly elevated following exposure to ozone (280% of control) and further augmented in a statistically significant manner in mice exposed to ADSS/ozone exposure, compared with all other groups. Since ETS and ozone represent the 2 most ubiquitous indoor and outdoor air pollutants, respectively, we wished to examine the effects of sequential exposure to ETS and ozone on patterns of lung injury. Studies to examine the interactive effects of ETS and ozone may help to illustrate the role of ETS in altering either the sensitivity to ozone injury or the pattern of inflammatory response of the lung. Such studies would be meaningful since exposure to air pollutants actually occurs as a complex mixture.

In the lung, alveolar macrophages (AM) serve as sentinels and are situated throughout from large bronchi to the alveolus. AM not only act as scavenger cells, they also secrete a whole array of bioactive factors including cytokines. TNF-α is among the early-response cytokines to appear at sites of inflammation. The production of TNF-α by alveolar macrophages can initiate the inflammatory response by promoting the production of chemotactic cytokines from a variety of cellular sources, all contributing to the recruitment of specific leukocyte subpopulations to sites of injury (Kunkel et al., 1998). IL-6 is a

Environmental pollutants forming a complex mixture of particles and gases can produce adverse health effects in people of all ages, particularly in young children and elderly individuals. Air pollutants may also serve to exacerbate pre-existing respiratory diseases (American Thoracic Society, 1996a,b). However, the mechanisms causing these effects remain in large measure unknown.

Environmental tobacco smoke (ETS), a common indoor air pollutant, is defined as the combination of sidestream smoke released from the burning end of the cigarette as well as mainstream smoke exhaled by the active smoker. Exposure to ETS is commonly referred to as passive smoking or involuntary smoking. Recently, ETS has been implicated as a significant risk factor for disease and increased morbidity and mortality among nonsmokers. Epidemiological studies have associated ETS exposure with a variety of health effects including cancer, coronary heart disease, sudden infant death syndrome, and reduced birth weight (NCI, 1999).

Ozone is a primary component of urban outdoor air pollution and is associated with heavy vehicular traffic. The action of sunlight on hydrocarbons and nitrogen oxides emitted in vehicle exhaust produces a complex oxidant mixture with ozone as a major product (American Thoracic Society, 1996a,b; Shy et al., 1991; van Bree et al., 1992). Ozone-induced pulmonary injury has been well investigated for more than three decades. The pattern of inflammatory response of the lungs to ozone has been extensively examined (Bhalla et al., 1987; Kehrl et al., 1987; Kleberger et al., 1990; Koren et al., 1989; Seltzer et al., 1986).

Since ETS and ozone represent the 2 most ubiquitous indoor and outdoor air pollutants, respectively, we wished to examine the effects of sequential exposure to ETS and ozone on patterns of lung injury. Studies to examine the interactive effects of ETS and ozone may help to illustrate the role of ETS in altering either the sensitivity to ozone injury or the pattern of inflammatory response of the lung. Such studies would be meaningful since exposure to air pollutants actually occurs as a complex mixture.

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pleiotropic cytokine that is produced at sites of tissue inflammation, thus implicating it as one of the proinflammatory cytokines. However, recent studies have shown that IL-6 has potent antiinflammatory and protective properties such as inhibiting the production of TNF, decreasing neutrophil sequestration, reducing intracellular superoxide production and inhibiting cellular apoptosis (Mizuhara et al., 1994; Rollwagen et al., 1998; Shingu et al., 1994; Ulich et al., 1991).

In this study, ADSS, composed primarily of sidestream smoke generated from 1R4F research cigarettes, was used as a surrogate of ETS. The exposure concentration of 30 mg/m³ of total suspended particulate matter (TSP) in this study was used to establish a worst-case scenario for exposure to ETS. Although this concentration may be as great as 250 times higher than the normal airborne particulate concentration of 120 μg/m³ detected in areas where smoking is permitted (Guerin et al., 1992), it is important to note that the cloud of particulates surrounding a person during active smoking can attain levels as high as 2 mg/m³ (EPA, 1992). Therefore, the concentration of smoke generated in this study, although high, may not be too far beyond what could occur under environmental conditions. The concentration of ozone selected in this study was 0.5 ppm. This concentration is equal to the atmospheric level for a stage-III smog alert in California (California Air Resource Board, 1990). Such a level of ozone, although uncommon, has been measured throughout the world, and most recently in Mexico City (Blake et al., 1995).

The purpose of this study was to evaluate the effects of prior exposure to ADSS on lung injury following subsequent exposure to ozone. We employed bronchoalveolar lavage and histologic evaluation of the centriacinar region, since this is a specific target site for the effects of ozone. We also examined the cellular response of leukocytes in lung air spaces, as well as the ability of AM to release tumor necrosis α (TNF-α) and IL-6. The ultimate goal was to investigate the potential role of TNF-α and IL-6 in lung sensitivity to injury.

MATERIALS AND METHODS

Exposure system. The exposure system used to generate aged and diluted cigarette smoke was originally described by Teague et al. (1994). Smoke was formed by burning filtered Kentucky 1R4F reference cigarettes in a smoking machine with a standardized 35-m1 puff volume of 2 s duration, once every min., for a total of 8 puffs per cigarette. Mainstream cigarette smoke, determined to contribute to 11% of total suspended particulate matter, was also directed into the sidestream cigarette smoke, which contributes to 89% of TSP (Witschi et al., 1998). Since ETS consists of a mixture of sidestream cigarette smoke as well as exhaled mainstream cigarette smoke, we felt mixing of both in these proportions would most closely approximate actual ETS exposure conditions. Sidestream and mainstream cigarette smoke were introduced into a conditioning chamber to dilute and age the cigarette smoke for a period of 2 to 4 min. To reach the desired target concentration, the cigarette smoke was further diluted with filtered air before it was introduced into the exposure chamber. Exposure conditions were monitored for carbon monoxide (CO) and nicotine, and total suspended particulate matter (TSP). TSP was determined by gravimetric measurement of samples collected from the chamber onto preweighed filters. CO was measured using an 880 model nondispersive-infrared (NDIR) analyzer (Beckmann Industries, La Habra, CA). Nicotine concentrations were measured by drawing air samples through sorbent tubes, extracting the nicotine, and performing analysis by gas chromatography (Ogden, 1989). Ozone was produced from vaporized, medical-grade liquid oxygen with a silent arc discharge ozonizer (Erwin Sander Corp., Giessen, Germany). Both the ozone and oxygen were conveyed through Teflon lines to the mixing inlet of the exposure chamber. Ozone concentrations in the chambers were monitored by Dasibi ultraviolet (UV) photometry calibrated against a standard reference photometer located at the California Air Resources Board Quality Assurance Laboratory. Data was recorded every 8 min directly into an IBM-AT computer for analysis. Each chamber was monitored for ozone concentration for a minimum of 15 min per h. Nominal exposure concentrations were within 5% of target values.

Cigarettes. Reference cigarettes (Kentucky 1R4F) were purchased from the Tobacco Research Institute, University of Kentucky, Lexington. The 1R4F is a filtered cigarette designed to deliver 11 mg tar and 0.8 mg nicotine per cigarette (Davis et al., 1984). The cigarettes were stored at 4°C until needed. At least 48 h prior to use, cigarettes were placed in a closed chamber at 23°C with a mixture of glycerin/water (mixed in a ratio of 0.76/0.24), in order to establish a relative humidity of 60%.

Animals. Male strain B6C3F1 mice (10 weeks of age, 25 ± 2 g body weight) were purchased from Charles River Laboratories (San Diego, CA). Random animals were selected for screening by the Comparative Pathology Laboratory at the University of California, Davis, to confirm the absence of viral and respiratory pathogens. A total of 44 mice were used in this experiment. During the course of the experiment, animals were housed 4 per cage, in polycarbonate boxes. The animals were maintained on a 12-h light/dark cycle and had free access to conventional laboratory feed and water. Animals were handled in accordance with standards established by the U.S. Animal Welfare Acts as set forth in the National Institutes of Health guidelines and by the University of California, Davis, Animal Care and Use Committee.

Exposure regimen. Animals were randomly divided into 4 groups, for exposure to filtered air, ADSS, ozone, or ADSS followed by ozone. In each group, 6 mice were used for bronchoalveolar lavage (BAL) and 5 mice were used for lung histology and cellular proliferation, using BrdU labeling. ADSS exposure was 6 h per day (from 9:00 A.M. to 3:00 P.M.) for 3 consecutive days. Concentrations of TSP, carbon monoxide, and nicotine in the ADSS chamber were 29.5 ± 0.5 mg/m³, 112 ± 3 ppm, and 8.9 ±1.1 mg/m³, respectively. Exposure to ozone was for 24 h at 0.5 ppm, beginning the following morning after exposure to ADSS. Exposure to ozone was from 9:00 A.M. to 9:00 A.M. the following day. During exposure to ADSS or ozone, control mice were housed in a chamber and exposed to filtered air. Mice used for BAL procedures were sacrificed within 2 h following ozone exposure, while mice used for histological studies were sacrificed 24 h after ozone exposure. Exposure protocols are shown in Figures 1 and 2.

Bronchoalveolar lavage. Mice were deeply anesthetized with sodium pentobarbital. The lungs were sequentially lavaged 4 times with identical aliquot volumes (35 ml/kg body weight) of Hank’s balanced salt solution (HBSS). Recovered aliquots of BAL fluid (BALF) were pooled together. BAL cells were pelleted by centrifugation at 500 × g for 10 min. The supernatant was used to measure total protein. The cell pellet was resuspended in 1 ml of phosphate-buffered saline (PBS). A volume of 100 μl of the cell suspension was mixed with 100 μl of 4% trypan blue (Gibaco, Grand Island, NY) to determine total cell number and viability. To determine the cell differential in BALF, 5 × 10³ cells were counted on a slide by cytospin centrifugation in 100 μl of HBSS at 100 rpm for 5 min, and were stained with Diff-Quik (Baxter Healthcare, Miami, FL). A total of 400 cells per animal were counted. Total protein in BALF was measured using a protein assay kit (Pierce, Rockford, IL).

Tissue preparation and immunohistochemistry. Alzet osmotic pumps (Alza Corporation, Palo Alto, CA) were implanted subcutaneously in mice one day before the start of exposure (Fig. 2). Each pump was filled with 200 μl BrdU solution (20mg/ml in phosphate balanced solution). Mice were sacrificed by intraperitoneal injection of an overdose of sodium pentobarbital one day before the start of exposure (Fig. 2). Each pump was filled with 200 μl BrdU solution (20mg/ml in phosphate balanced solution). Mice were sacrificed.
after ozone exposure was completed. The lungs were inflation fixed in situ by intratracheal instillation of 4% paraformaldehyde for 1 h. Lung tissues were transferred into 75% ethanol, dehydrated in 95% and 100% ethanol, and embedded in paraffin. The blocks were cut on a rotary microtome into 5-μm sections and mounted on superfrost-plus glass slides (Fisher Scientific, Pittsburgh, PA). For immunohistochemistry, alkaline phosphatase-conjugated anti-BrdU monoclonal antibodies (Roche, Minneapolis, MN) were used. BrdU-positive cells were visualized using 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT-BCIP) as the substrate, following the manufacturer’s protocol. All sections were counterstained with nuclear fast red. As a positive control for BrdU immunolabeling, all slides included a section of small intestine.

**Evaluation of cell labeling.** All slides were coded and counted without knowledge of the exposure regimen for each group. Labeling indices were determined by counting a total of 1000 cells within the centriacinar region of each animal. The centriacinar region was identified as the bronchiole-alveolar duct junction and included both the terminal bronchiole and proximal alveolar regions (Fig. 3). Only terminal bronchioles that were continuous with the proximal alveolar regions in the section were used to determine the labeling index. The BrdU labeling index was calculated as the percentage of BrdU-labeled cells over total cell number. Counting of cells was done at ×400 magnification.

**Analysis of cytokine release by BAL recovered alveolar macrophages.** AM were recovered by BAL. Cells were separated from BALF supernatant by centrifugation and cell pellets were resuspended in RPMI-1640 medium. Approximately 10^5 cells per mouse were seeded into each well of a 48-well plate. After incubation for 30 min at 37°C in a 5% CO2 atmosphere, nonadherent cells were rinsed from the wells. Complete RPMI-1640 medium, 0.8 ml supplemented with antibiotics and 10% fetal bovine serum (FBS), was added to each well. To determine LPS-stimulated cytokine release, LPS was added into the medium at a final concentration of 20 μg/ml. After incubation for 20 h at 37°C in 5% CO2 atmosphere, the supernatant was removed and stored at −80°C until ready for analysis. Enzyme-linked immunosorbent assays (ELISA) of IL-6 and TNF-α were done using OptEIA® ELISA kits from PharMingen (San Diego, CA).

**Statistical analysis.** All data are expressed as mean ± 1 standard deviation (SD). Statistical comparisons between groups were made using ANOVA (StatView 4.5, Abacus Concepts, Inc., Berkeley, CA). Multiple comparisons between all groups were performed by Fisher’s least-significant-difference test. A p value of 0.05 or less was considered to be significant.

**RESULTS**

Total cell number, cell viability, and protein level in BAL are shown in Table 1. Exposure to ADSS followed by ozone was associated with a statistically significant increase in total cell number compared with control animals. Exposure to ADSS alone or ozone alone did not significantly change total cell number. Total protein in BALF was found to be significantly increased in animals exposed to ADSS followed by ozone (p < 0.05). This increase in protein level was also significantly higher than that found in animals exposed to ozone alone.

In mice exposed to filtered air only, over 96% of cells recovered by BAL were macrophages and 2% were monocytes. In contrast, the percentage of monocytes following exposure to ADSS alone, ozone alone, or ADSS/ozone was significantly elevated. The percentage of neutrophils in the animals exposed to ozone was also significantly increased and further increased by prior exposure to ADSS with subsequent exposure to ozone. Prior exposure to ADSS followed by exposure to ozone also significantly increased the percentage of lymphocytes in BALF (Table 2).

Labeling with BrdU throughout the lung airways and parenchyma demonstrated that the greatest proportion of positive cells were within the centriacinar regions (Fig. 3). No significant alteration in cell proliferation was observed in other sites of the lungs. Compared with controls, the labeling index of BrdU within the centriacinar regions of the lungs was un-
changed following exposure to ADSS alone. In contrast, exposure to ozone caused a 3-fold increase in BrdU labeling within the centriacinar region, while exposure to ADSS followed by exposure to ozone was associated with a 4-fold increase in BrdU labeling within this region (Fig. 4).

Measurement of IL-6 and TNF-α levels in the cell culture supernatant of AM showed that exposure to ozone alone or ADSS plus ozone significantly decreased the spontaneous release of IL-6 (Fig. 5). LPS-stimulated IL-6 release by AM recovered from animals exposed to ozone alone or ADSS followed by ozone followed a similar pattern.

The ability of AM to release TNF-α was also affected by the type of exposure. A significant decrease in the spontaneous production of TNF-α was found in AM from animals exposed to ozone alone or to ADSS/ozone (Fig. 5). Interestingly, when AM were stimulated with LPS in culture, the levels of TNF-α released in the supernatant from animals exposed to ADSS or ADSS/ozone were both significantly increased compared with filtered-air-exposed control animals.

**DISCUSSION**

This study demonstrates striking effects of exposure to ADSS to enhance pulmonary injury caused by subsequent exposure to ozone. It was also found that prior exposure to ADSS altered the inflammatory cell profile in the lungs, as well

**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total recovered cells ($10^5$)</th>
<th>Viability (%)</th>
<th>Protein level ($\mu$g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtered air</td>
<td>3.58 ± 2.68</td>
<td>95.2 ± 3.6</td>
<td>548 ± 108</td>
</tr>
<tr>
<td>ADSS</td>
<td>3.56 ± 1.84</td>
<td>88.9 ± 4.5</td>
<td>752 ± 306</td>
</tr>
<tr>
<td>Ozone</td>
<td>2.98 ± 1.75</td>
<td>86.2 ± 4.5</td>
<td>848 ± 139</td>
</tr>
<tr>
<td>ADSS/Ozone</td>
<td>6.44 ± 2.68***</td>
<td>85.5 ± 10.6</td>
<td>1219 ± 290***</td>
</tr>
</tbody>
</table>

*p < 0.05, compared with filtered group, n = 6.
**p < 0.05, compared with ozone group, n = 6.

**TABLE 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Monocyte (%)</th>
<th>Macrophage (%)</th>
<th>Lymphocyte (%)</th>
<th>Neutrophil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtered air</td>
<td>2.2 ± 0.9</td>
<td>96.9 ± 0.9</td>
<td>0.6 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>ADSS</td>
<td>4.2 ± 0.7*</td>
<td>94.7 ± 0.7</td>
<td>0.5 ± 0.2</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Ozone</td>
<td>4.5 ± 0.6*</td>
<td>91.1 ± 2.8*</td>
<td>0.5 ± 0.3</td>
<td>4.0 ± 2.2*</td>
</tr>
<tr>
<td>ADSS/ozone</td>
<td>3.4 ± 0.4*</td>
<td>88.1 ± 2.3*</td>
<td>1.1 ± 0.4*</td>
<td>7.5 ± 2.5*</td>
</tr>
</tbody>
</table>

*p < 0.05, compared with filtered group; n = 6.
**p < 0.05, compared with ozone group; n = 6.
As changing the response of AM to produce cytokines following subsequent exposure to ozone.

Incorporation of 5-bromo-2′-deoxyuridine (BrdU), a thymidine analogue, into DNA during DNA replication is a frequently utilized method for estimating cell proliferation (Ayres et al., 1995; Doolittle et al., 1992). Cell proliferation plays a critical role in restoring a normal lung balance following injury. A proliferative response in the lungs is thought to reflect injury and death of cells (Evans et al., 1978, 1982; Kauffman et al., 1980). Due to its oxidizing properties, ozone is able to react with mucosal membranes, which causes structural and functional changes. Ozone-induced lung injury has been well-established in animals as a model. Cell proliferation within the centriacinar regions of the lungs is a typical pattern of the process of repair following ozone injury (Pinkerton et al., 1992; Rajini et al., 1993). A number of studies have demonstrated that disruption of the epithelial barrier results in increased pulmonary permeability following exposure to varying ozone concentrations (reviewed by Bhalla, 1999). In the present study, an elevated BrdU labeling index was present within the intrapulmonary airways or centriacinar regions in male B6C3F1 mice exposed to 30 mg ADSS/m³ for 3 days compared with control animals exposed to filtered air. A parallel study using male A/J mice also confirmed this same pattern of BrdU labeling in the lung following exposure to cigarette smoke (Yu et al., 1999). Whether this difference between rats and mice is due to the species, exposure protocol, or BrdU labeling technique has not been resolved. In the present study, we also found total cell number and total protein level in BALF were unchanged following exposure to ADSS, suggesting little to no inflammatory response present in the lungs of mice following exposure to cigarette smoke under the conditions used.

After an extensive review of studies involving ozone-induced lung inflammation and injury, Bhalla concluded that a compromised epithelial barrier induced by prior exposure to ADSS labeling within the intrapulmonary airways or centriacinar regions in male B6C3F1 mice exposed to 30 mg ADSS/m³ for 3 days compared with control animals exposed to filtered air.

There is limited information available on lung injury caused by exposure to ETS or ADSS used as a surrogate of ETS. Ayres and colleagues conducted experiments to expose male Sprague-Dawley rats to 0.1, 1, or 10 mg ADSS/m³ for 6 h/day, 5 days/week, for a period of 5, 28, or 90 days, followed by examination of BrdU labeling in bronchial, bronchiolar, and alveolar cells of the lungs. No significant increase in BrdU labeling was observed, except in airway bronchiolar cells, following exposure to 10 mg ADSS/m³ for 5 days (Ayres et al., 1995). Our study showed no significant difference in BrdU labeling within the intrapulmonary airways or centriacinar regions in male B6C3F1 mice exposed to 30 mg ADSS/m³ for 3 days compared with control animals exposed to filtered air.

**FIG. 4.** BrdU labeling index within the centriacinar regions of the lungs. The labeling index is expressed as the proportion of BrdU immunolabeled positive cells to the total cell number counted within the centriacinar regions of the lungs; *p < 0.05 compared with filtered air control. †p < 0.05 compared with animals exposed to ozone alone.

**FIG. 5.** Levels of IL-6 and TNF-α in the culture supernatant of AM. From each animal, 10⁵ cells recovered by BAL in 0.8 ml complete RPMI-1640 medium were seeded in each well of a 48-well plate. Following incubation for 20 h at 37°C in a 5% CO₂ atmosphere, in the absence of LPS (open bar) or the presence of LPS (filled bar), at a final concentration of 20 μg/ml, the culture supernatant was harvested and cytokine levels were determined using an ELISA method; *p < 0.05 compared with filtered air control, †p < 0.05 compared with animals exposed to ozone alone.
environmental pollutants, no matter how transient, introduces an additional risk factor by predisposing the lung to further injury with further exposure to environmental toxicants (Bhalla, 1999). In the present study, it has been clearly demonstrated that prior exposure of animals to ADSS sensitizes the lungs to greater ozone-induced injury reflected by the potentiated effect of BrdU labeling within the centriacinar regions of the lungs following subsequent exposure to ozone. This pre-exposure to ADSS significantly enhanced BrdU labeling in mice following exposure to ozone, and was also accompanied by a significant increase in the recruitment of leukocytes into the lung air spaces as well as by a significant elevation in total protein in BALF, compared with mice exposed only to ozone.

Resident macrophages in the lungs play an important role in pulmonary inflammation. They are in intimate contact with epithelial surfaces. An exogenous challenge can induce a significant response in AM to release cytokines or other mediators, which may be involved in the initiation, maintenance, or resolution of the inflammatory reaction involving the epithelial lining of the lungs (Berman et al., 1998). Studies of cytokine profiles of AM induced by prior exposure to ADSS may help to elucidate the potential pathway by which ADSS contributes to increased lung sensitivity to ozone injury. For cells in an in vitro culture system, it is unlikely that AM will perform as they do in vivo. In the present study, the ability of AM to release cytokines was found to be significantly enhanced by stimulation with LPS (t test, p < 0.05, respectively), regardless of the treatment group from which they were recovered. A different pattern of TNF-α induction was also observed in LPS stimulated AM compared with unstimulated AM. We propose that the ability of AM to release cytokines under LPS stimulation may better reflect their actual potential to respond under adverse environmental influences. The plausibility of this concept is further based on experiments by Hasday and coworkers (Hasday et al., 1999) who found bioactive LPS to be present in mainstream smoke (120 ± 64 ng/cigarette) and sidestream smoke (18 ± 1.5 ng/cigarette) from 1R4F reference cigarettes identical to those used in the present study to generate tobacco smoke.

A significant elevation in TNF-α production by AM following exposure to ADSS may play a critical role in the increased sensitivity of lungs to ozone injury. TNF-α could initiate apoptotic signaling by binding to TNF receptor I (TNFR I) on the surfaces of alveolar epithelial cells (Kuebler et al., 2000). TNF-α has also been found to increase the expression of c-Jun (Bohler et al., 2000; Tengku-Muhammad et al., 2000), and to induce the activation of c-Jun N-terminal protein kinase (JNK) and activator protein (AP)1 to promote the apoptotic process (Manna et al., 2000). Furthermore, TNF-α is involved in the initiation of apoptotic signaling by increasing the surface expression of Fas on epithelial cells (Matsumura et al., 2000; Trifilieff et al., 1999). Binding of TNF-α to TNFR escalates intracellular production of reactive oxygen species (ROS) and increased mitochondrial superoxide anion production, followed by the release of cytochrome c and caspase-3 activation, which culminate in apoptotic cell death (Garcia-Ruiz et al., 2000).

Cells cultured in media conditioned with tobacco smoke can be induced to express hsp70 and Bcl-2 proteins that protect cells from apoptosis by preventing the release of cytochrome c from mitochondria, thus inhibiting caspase-3 activation and blocking of JNK activation (Gabai et al., 1997; Jager et al., 1997; Li et al., 2000; Trump et al., 1998; Vayssier et al., 1998). This raises the possibility that ADSS exposure could induce Bcl-2 and/or hsp70 expression in respiratory epithelial cells, which may contribute to the inhibition of apoptotic cell death. If these protective processes are activated in the lungs following ADSS exposure, apoptosis should not be present. Typically, apoptosis in the lungs would be followed by cell proliferation, which can be easily assessed by an elevation in the BrdU labeling index of the lungs.

An interesting report by Kirichenko and colleagues (1996) has documented that a toxic, oxidant-induced lipid peroxide, 4-hydroxy-2-nonenal (HNE), is formed in the lungs following exposure to ozone. It is highly plausible that this peroxide could inhibit an upstream component of the NF-κB signaling pathway that acts by inducing the expression of antiapoptotic proteins, including Bcl-2 and manganese superoxide dismutase (Camandola et al., 2000). Recently, the expression of hsp70 (as well as that of other members of the hsp family) by respiratory epithelial cells in the lung can be decreased by exposure to ozone (Wu et al., 1999). These studies support the tenet that exposure to ozone could deplete mechanisms serving to protect ADSS-exposed cells from apoptosis. Thus, the depletion of those self-protective mechanisms by exposure to ozone will drive ADSS-affected cells to a final and irreversible stage of apoptotic cell death. Such a combination of events could contribute to the observed potentiated effect of prior exposure to ADSS to enhance BrdU labeling within the centriacinar regions in the lungs, following subsequent exposure to ozone.

The role of IL-6 in the inflammatory response remains controversial. Increased IL-6 expression in BALF is typically associated with lung injury (Cuzzocrea et al., 1999; Jorres et al., 2000). However, it has been reported that direct exposure of AM to ozone in vitro does not cause an increase in IL-6 release (Devlin et al., 1994). In the present study, the ability of AM in vitro to release IL-6 was significantly inhibited in animals exposed to ozone. Prior exposure to ADSS followed by exposure to ozone was associated with even lower levels of IL-6 in the culture supernatant of AM. It is interesting to note that a decreased level of IL-6 in the culture supernatant of AM was also associated with a decrease in the percentage of AM recovered in BALF. Mitani and colleagues (2000) found IL-6 could promote the differentiation of monocytes toward macrophages. Therefore, decreased release of IL-6 by AM may be correlated with an increased percentage of monocytes as well
as a decreased percentage of AM in BALF. Since AM are not the only source of IL-6 produced in the lung, the contribution of AM-derived IL-6 to cell proliferation within the centriacinar regions is uncertain. On one hand, IL-6 can protect cells from injurious stimuli via the inhibition of apoptosis (Teague et al., 1997; Ogawa et al., 2000), which may contribute to less BrdU labeling. On the other hand, IL-6 is involved in tissue repair and supports cell proliferation (Liechty et al., 2000; Sakamoto et al., 1999; Sato et al., 2000), which results in more BrdU labeling. Experiments are currently being conducted in our laboratory to examine IL-6-deficient mice, following the identical protocol used in this study, as a possible means to clarify the role of IL-6 in this proliferative response.

In summary, exposure of mice to ADSS alone does not cause significant changes in pulmonary permeability or cell proliferation in the lungs. However, prior exposure of mice to ADSS is associated with a significantly enhanced sensitivity of the lungs to subsequent exposure to ozone, resulting in more severe lung injury. Alteration of pulmonary leukocyte profiles as well as the cytokine response of AM may contribute to this increased sensitivity to lung injury. Further research should be done to elucidate the role of AM and AM-derived cytokines in the signaling of apoptotic cell death in the lungs to better understand the mechanism of ADSS-induced sensitivity of the lungs to ozone injury.

ACKNOWLEDGMENTS

This work was funded in part by the National Institutes of Health (ES05707 and RR00169), as well as the California Tobacco-Related Disease Research Program (6RT-0327 and 7RT-0118).

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