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Amelioration of Ozone-Induced Lung Injury by Anti-Tumor Necrosis Factor-α

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Ozone (O_3) is a significant component of atmospheric air pollution and produces detrimental effects in the lung. Although the mechanism of O_3-induced lung inflammation and injury is unclear, the increased release of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) by lung cells following O_3 exposure may shed some light on this subject. To investigate the role of TNF-α in the O_3-induced pulmonary insult, we intraperitoneally injected rats with either rabbit preimmune serum or rabbit antirat TNF-α 1 h prior to O_3 exposure. Approximately 12 h after the end of O_3 exposure the animals were sacrificed, the lungs lavaged, and tissue samples collected for expression of cytokine genes relevant to inflammation. The bronchoalveolar lavage fluid (BALF) was analyzed for albumin as a marker of pulmonary epithelial permeability changes and for fibronectin for its role in lung injury and repair. The lavage cells were collected, counted, and identified to quantitate the inflammatory response. Ozone exposure resulted in a significant increase in BALF albumin and fibronectin as compared to air-exposed controls and a significant increase in BALF polymorphonuclear leukocytes (PMNs). Antibody treatment produced a significant decrease in BALF albumin and PMNs as compared to O_3-exposed rats given preimmune serum. Antibody treatment did not affect the BALF fibronectin concentration or the total cell count in the BAL. Tissue analysis for gene arrays revealed an activation of IL-1α, IL-6, and IL-10 in animals exposed to O_3. The gene expression was downregulated in animals treated with anti-TNF-α antibody prior to O_3 exposure. The results suggest a central role for TNF-α in the mechanistic pathways critical to lung inflammation. The significance of TNF-α in the inflammation and epithelial injury produced by ozone exposure reflects its overall contribution through modulation of other cytokines.

Key Words: **tumor necrosis factor-α; permeability; lung; inflammation; cytokines; rats.**

Ozone (O_3), an oxidizing gas, is a significant component of air pollution. Exposure to supra-ambient levels of O_3 results in lung inflammation and injury in both humans and laboratory animals. This is characterized by a significant increase in the percentage of polymorphonuclear leukocytes (PMNs) in the bronchoalveolar lavage fluid (BALF; Bassett *et al.,* 1988; Koren *et al.,* 1989; Pino *et al.,* 1992) and perturbations of the epithelial tight junctions allowing serum proteins and albumin into the air spaces (Bhalla, 1999; Bhalla *et al.,* 1999). At the cellular level, *in vitro* exposure of cultured cells to O_3 results in the release of the proinflammatory cytokine tumor necrosis factor-α (TNF-α). This has been observed with guinea pig alveolar macrophages, human alveolar macrophages (Arsalane *et al.,* 1995), and human bronchial epithelial cells (Rusznak *et al.,* 1996), while the *ex vivo* release of TNF-α following O_3 exposure has been reported in macrophages (Pendino *et al.,* 1994). Unfortunately, the role of this proinflammatory cytokine in the pathophysiology of O_3-induced lung injury remains relatively undefined.

The purpose of our study was to determine the contribution of TNF-α in the development of O_3-induced lung injury by the administration of anti-TNF-α prior to O_3 exposure. Because the focus of this study was on inflammatory mechanisms, genes relevant to inflammation were analyzed using gene array, a pathway-specific gene expression profiling system, to identify the molecular pathways activated in response to O_3 exposure. The focused approach in this study allowed us to concentrate on a select group of cytokines, without having to perform microarray analyses commonly used to screen hundreds or thousands of genes. At the same time, this procedure allowed analysis of over 20 genes simultaneously using a single hybridization assay, thus presenting an advantage over the conventional procedures, such as RT-PCR and Northern blot analyses, which detect the expression of one gene in a single assay. Activation of cytokine genes as a function of TNF-α status identified a central role of this cytokine in modulating events critical to inflammation.

MATERIALS AND METHODS

Animals. Specific pathogen-free male Sprague-Dawley rats (250–275 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and shipped by the supplier in filtered containers. Upon arrival, the animals were housed in polycarbonate cages and kept in a laminar flow caging system until used for the study. The animals were quarantined for one week prior to use to allow

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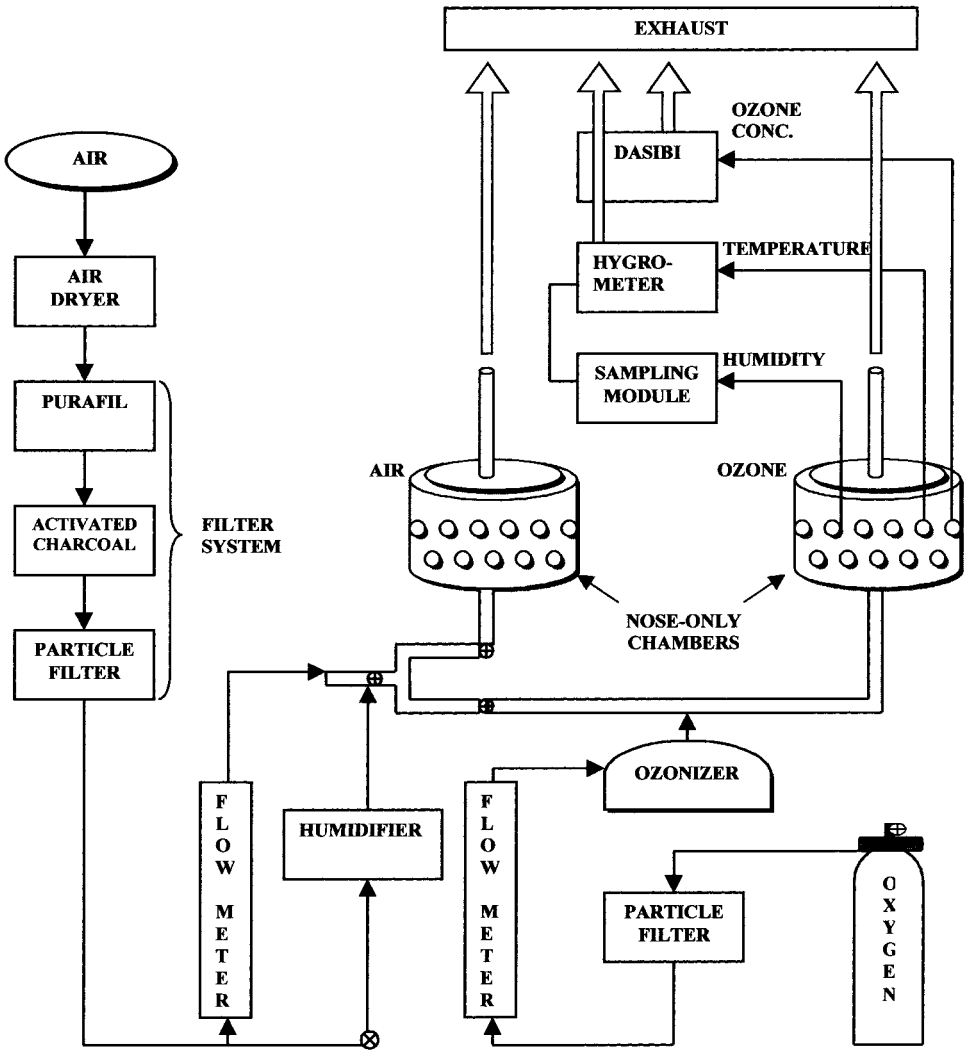

FIG. 1. Inhalation exposure system for nose-only exposure of rats to air or ozone.

acclimation to the facility and to detect any transportation and stress related illnesses. The vivarium was AAALAC (American Association for the Accreditation of Laboratory Animal Care) accredited and the rats were maintained in accordance with the guidelines of the University Committee for Animal Care, receiving food and water *ad libitum*.

Female White New Zealand rabbits were purchased from Harlan Sprague-Dawley, housed individually in steel wire cages, and quarantined for one week prior to use in the study. The antigen consisted of recombinant rat TNF-α (Biosource International, Camarillo, CA) diluted in sterile phosphate-buffered saline (PBS) to a concentration of 20 μg/ml and mixed 1:1 with Freund's Complete Adjuvant to make a total of 2 ml. The rabbits were anesthetized with acepromazine (0.75 mg/kg) and xylazine (5 mg/kg) im and multiple intradermal injections were given using a volume of 100 μl. The remaining antigen solution (0.5 ml) was given ip. Booster injections were given two and four weeks later in the same manner as above using an antigen concentration of 10 μg/ml, then mixing with Freund's Incomplete Adjuvant. Blood was drawn every two weeks and the animals exsanguinated when antibody titers reached a plateau. The antibody was purified by incubating with Protein A and the purified IgG was used in the study.

Experimental design. The optimal rabbit antirat TNF-α IgG dose was derived from pilot studies in which rats received a series of antibody doses prior to O_3 exposure. A dose of 20 mg/kg provided maximum protection while minimizing inflammation. For the studies described in this article, rats were given ip injections (20 mg/kg) of either the rabbit antirat TNF-α IgG or preimmune rabbit IgG. Approximately 1 h later, the rats were exposed for 3 h to either filtered air or O_3 (1 ppm) using a nose-only exposure system. The rats were returned to their cages and 10–12 h later sacrificed. The selection of this time point was based on the results of our previous studies (Bhalla *et al.*, 1999) showing peak increases in BAL markers of injury and inflammation at 8 to 12 h after the end of O_3 exposures comparable to those used in this study. The lungs were lavaged and the BALF analyzed for albumin content. The lavage cells were counted using a coulter counter and identified morphologically. For tissue analyses, lungs were rapidly removed, frozen in liquid nitrogen, and stored at −80°C.

Animal exposure. Air and O_3 exposures were performed using a nose-only exposure system (Fig. 1). The inhalation chambers were purchased from CH Technologies (Westwood, NJ) and the exposure system was assembled in our laboratory. The chambers are designed to permit delivery of atmospheres to individual rats so that air exhaled by one animal is not inhaled by another animal. The chamber atmosphere was maintained at an air temperature of 75.7 $\pm$ 0.1°F, an O_3 concentration of 1.018 $\pm$ 0.003 ppm, and a relative humidity of 45.5 $\pm$ 0.5%. The chambers were supplied with dry air that had

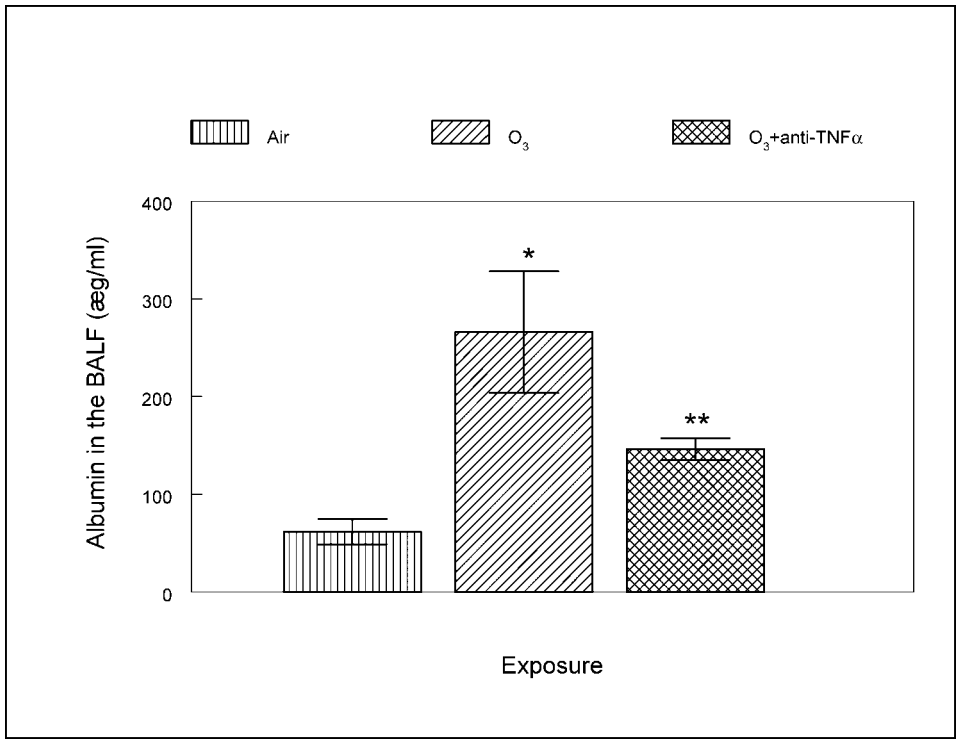

FIG. 2. The effect of O_3 exposure and anti-TNF-α treatment on the albumin levels in the BALF. Control animals received preimmune rabbit IgG prior to exposure. The results represent the group mean ± SEM. *Significant increase compared to the air-exposed controls. **Significant decrease compared to the O_3 group, $p \leq 0.05$.

been passed through activated charcoal, purafil, and a high performance filter before being humidified to the desired level. Ozone was generated by passing medical grade oxygen through an electrical O_3 generator (Sander Ozonizer, Osterberg, Germany) and injecting it into the airstream. Ozone concentration was continuously monitored at the rat breathing zone using a calibrated ultraviolet light absorption monitor (Dasibi Environmental Corporation, Glendale, CA). The air temperature and relative humidity were continuously monitored using a hygrometer (EdgeTech, Milford, MA).

Bronchoalveolar lavage. The animals were anesthetized with an ip injection of sodium pentobarbital (50 mg/kg) and the tracheas were cannulated with polyethylene tubing. The abdomen was opened and the animals exsanguinated by severing a major vessel. The diaphragms were dissected to allow free lung expansion and the lungs were lavaged by slowly instilling 8 ml of warm PBS (Ca^{++} and Mg^{++} free, 37°C) then gently aspirating. This cycle was repeated three times using the same volume and BALF was collected and centrifuged; supernatant was stored at –70°C. The lavage cells were quantified electronically, and cell differentials were determined by examining cytospin cell smears stained with Diff Quik (Baxter Healthcare Corporation, McGraw Park, IL). Trypan blue exclusion revealed greater than 90% viability in all samples.

Analysis of albumin. The concentration of albumin in the BALF was determined colorometrically using the Sigma Diagnostics Albumin Reagent. Briefly, 100 μl of BALF was pipetted in triplicate into the appropriate wells of a 96 well round-bottomed plate. Standards consisted of dilutions of rat albumin in PBS. This was followed by the addition of 100 μl Sigma Diagnostics Albumin Reagent. The absorbance was immediately measured at 620 nm and the concentration of albumin in the samples was calculated from the standard curve.

Analysis of fibronectin. The concentration of fibronectin in the BALF was determined by ELISA as described by Gomez-Lechon and Castell (1985). Briefly, high protein-binding multiwell plates were coated overnight with 100-fold dilutions of BALF samples. After washing, the wells were incubated with goat antirat fibronectin as the primary antibody. Secondary antibody consisted of rabbit antigoat IgG conjugated with horseradish peroxidase. Color was developed with o-phenylenediamine HCl and the fibronectin levels determined from the standard curve.

Nonradioactive gene array. Total tissue RNA was extracted from rat lungs using Qiagen RNeasy Midi Kit (Qiagen Inc., Valencia, CA). RNA integrity was examined by running an RNA gel. Gene array procedure followed a Nonrad-GEArray Kit manual (Superarray Inc., Bethesda, MD). In brief, biotinylated cDNA probe was synthesized by reverse transcription at 42°C by mixing GEA primer mix with dNTP containing biotin-dUTP. Biotinylated cDNA probes were hybridized to gene-specific cDNA fragments spotted on the membranes in hybridization oven overnight at 68°C. GEArray membranes were washed 4 times, blocked with GEA blocking solution, and then incubated with alkaline phosphatase conjugated streptavidin (1:5000). After sequential washes the membranes were incubated with 5 ml CDP-Star chemiluminescent substrate for 2 min and exposed to X-ray film. The exposures were performed for varying lengths of time to determine the abundance of different transcripts. The films were scanned by Personal Densitometer (Molecular Dynamics Inc.) to obtain densitometric volumes.

Statistics. Results for albumin, fibronectin, and cell count were obtained from 6 rats per exposure/treatment group and expressed as means ± SE. Comparisons between multiple groups were analyzed using Tukey's test with a probability of < 0.05 considered significant.

RESULTS

Bronchoalveolar Permeability

To assess the changes in bronchoalveolar permeability, the albumin levels in BALF of each rat were analyzed. Ozone exposure resulted in a significant elevation of the BALF albumin levels as compared to air-exposed controls. This increase was significantly diminished in the group receiving anti-TNF-α prior to O_3 exposure (Fig. 2).

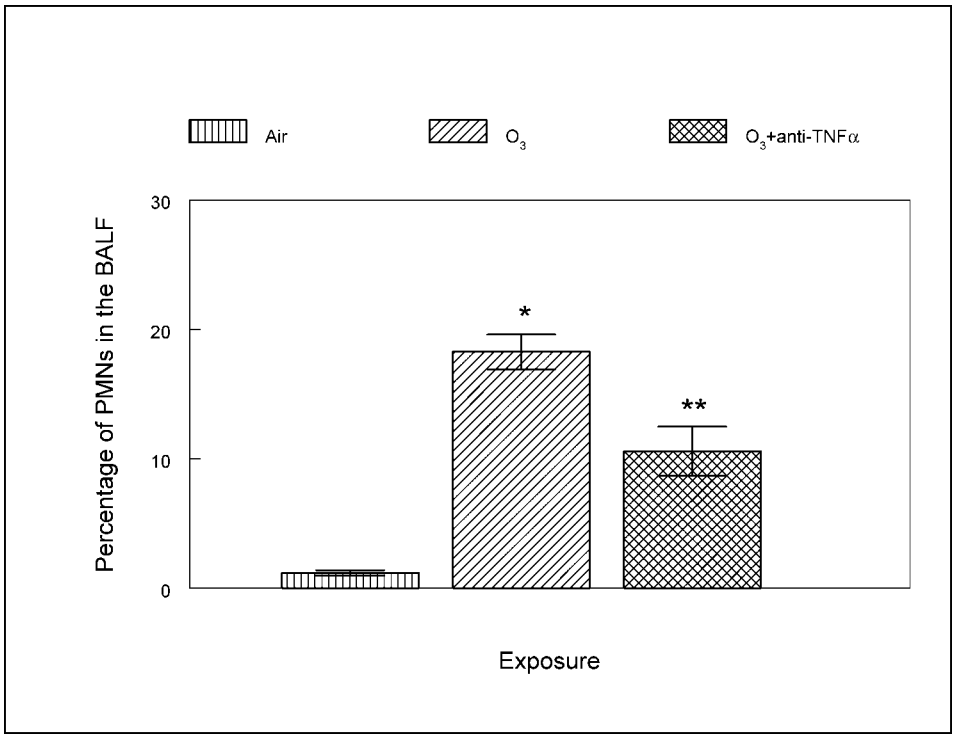

FIG. 3. Polymorphonuclear leukocytes (PMN) recovered in the BALF samples from animals exposed to O_3 or treated with anti-TNF-α prior to O_3 exposure. Control animals received preimmune rabbit IgG prior to exposure. The data are % PMN mean ± SE. *Significant increase compared to the air-exposed controls. **Significant decrease compared to the O_3 group, $p \leq 0.05$.

Inflammatory Response

To characterize the pulmonary inflammatory response in the various groups, BALF cell differentials were determined from stained cytospin preparations. Ozone exposure resulted in a significant increase in PMNs as compared to controls. The group receiving anti-TNF-α prior to O_3 exposure had significantly fewer PMNs in the BALF as compared to O_3 exposure

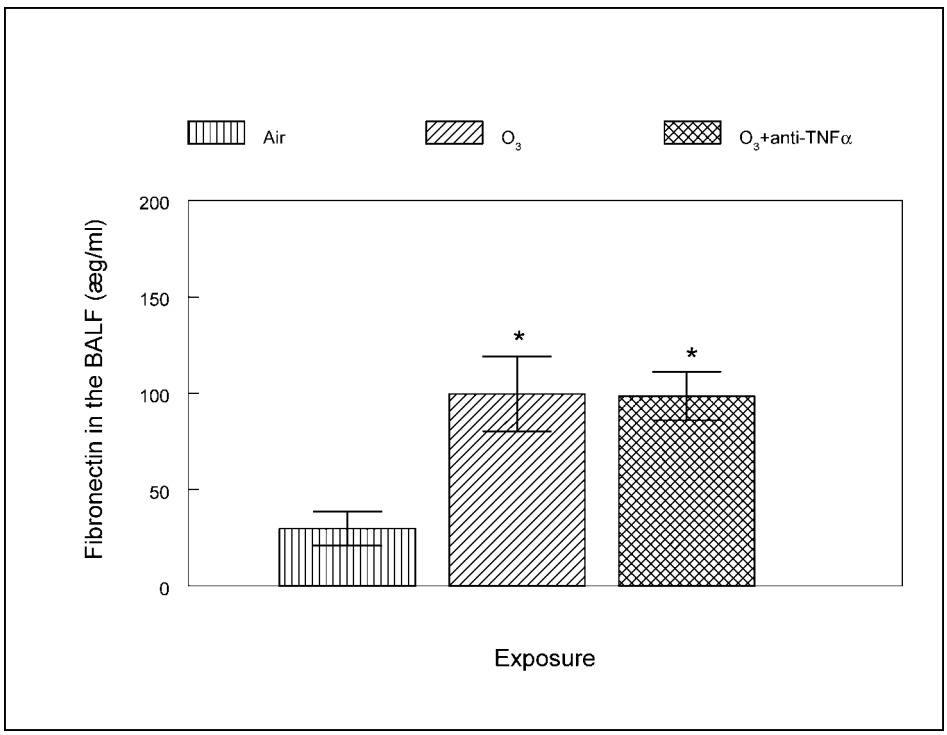

FIG. 4. The effect of O_3 exposure and anti-TNF-α treatment on the fibronectin levels in the BALF. Control animals received preimmune rabbit IgG prior to exposure. The results represent the group mean ± SEM. *Significant difference between air-exposed controls and experimental groups, $p \leq 0.05$.

TABLE 1
Cytokine Genes Included in the Selected Gene Array

Gene name	Description
G-CSF	Colony stimulating factor, granulocyte
Gro 1	GRO 1 oncogene
IL-1α	Interleukin 1α
IL-1β	Interleukin 1β
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5 (colony-stimulating factor, eosinophil)
IL-6	Interleukin 6 (interferon, β2)
IL-10	Interleukin 10
IL-12a	Interleukin 12A
IL-12b	Interleukin 12B
IL-16	Interleukin 16
IL-17	Interleukin 17
IL-18	Interleukin 18
LT-b	Lymphotoxin B
MIF	Migration inhibitory factor
MCP-1	Macrophage chemoattractant protein-1
TGF-α	Transforming growth factor-α
TGF-β1	Transforming growth factor-β1
TGF-β2	Transforming growth factor-β2
TGF-β3	Transforming growth factor-β3
TNF-α	Tumor necrosis factor-α
TNF-β	Tumor necrosis factor-β
β-actin	β-actin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
pUC18	Bacterial plasmid

alone (Fig. 3). In contrast, no significant differences in BALF total cell counts were noted between any of the groups (air: 2.9 $\pm$ 0.3; ozone: 3.6 $\pm$ 0.9; ozone + antibody: 2.8 $\pm$ 0.4 [expressed as 1×10^6 cells]).

Fibronectin Analyses

Significant increases in BALF fibronectin were found in the group exposed to O_3 alone as compared to air-exposed controls. Pretreatment with anti-TNF-α did not prevent the O_3-induced increase in BALF fibronectin concentration. The levels of fibronectin were comparable between the anti-TNFα + O_3 and O_3 alone groups (Fig. 4).

Gene Expression Profile

Although microarrays are used to screen a large number of genes, the gene array used in this study permitted us to focus on a smaller set of genes relevant to inflammation. Selected tissue samples were analyzed for cytokine gene expression mainly to support ELISA results. Table 1 lists the cytokine genes included in the selected gene array. The gene array format on hybridization membrane is shown in Figure 5A. Rat lungs from control, O_3 exposure, and O_3 plus antibody groups were collected and total RNA extracted to define the gene expression profile (Figs. 5B, 5C, and 5D). These gene array blots were scanned and the densitometric volumes were recorded. The results presented in Figure 6 represent genes that were differentially expressed following O_3 exposure and O_3 + anti-TNF-α treatment. These included genes for TNF-α, IL-1α, IL-6, and IL-10. These 4 genes were expressed at a higher level in lungs from rats exposed to O_3 compared to those from the air-exposed controls, and their expression levels were lower in rats treated with anti-TNF-α antibody prior to O_3 exposure.

DISCUSSION

The acute inhalation of O_3 results in a transient pulmonary inflammation with concomitant permeability changes resulting in the leakage of serum proteins into the air spaces. In addition, the expressions of TNF-α are increased. The significance of the release of TNF-α as it relates mechanistically to O_3-induced lung inflammation and injury is not fully understood. Therefore the aim of our study was to investigate the role TNF-α plays in this injury with regard to the changes in lung epithelial permeability and the inflammatory response.

The lung epithelium serves as a barrier to prevent entry of serum proteins into the air spaces and inhaled xenobiotics from gaining access to the vascular system. Ozone is known to disturb the tight junctions between cells allowing serum proteins to enter the air spaces (Bhalla, 1999). Albumin being one of the major serum proteins serves as an indicator of epithelial permeability and can be quantitated in the BALF. Collectively, permeability changes arising from O_3 exposure may be the result of both direct injury to the cellular tight junctions and indirectly from the release of TNF-α. The administration of anti-TNF-α prior to O_3 exposure in our study resulted in a significant reduction in BALF albumin as compared to O_3 alone. Similar results have been reported from ischemia/reperfusion injury models (Caty *et al.*, 1990; Colletti *et al.*, 1990; Seekamp *et al.*, 1993) and in gram-negative sepsis (Windsor *et al.*, 1994). The mechanism by which TNF-α causes permeability changes has received considerable attention. Depletion of granulocytes by cyclophosphamide in guinea pigs has been shown to prevent the TNF-α induced lung injury (Stephens *et al.*, 1988). In addition, Goldblum and colleagues (1989) have shown that TNF-α is capable of producing *in vitro* as well as *in vivo* injury to pulmonary vascular endothelium. Albumin transfer across cultured porcine pulmonary artery endothelial cell monolayers was increased by TNF-α in the absence of granulocytes (Goldblum *et al.*, 1989).

Ozone exposure also resulted in a significant increase in PMNs recovered in the BALF. In contrast, the administration of TNF-α antibody resulted in a significant reduction in PMNs. This is consistent with the findings of Windsor and colleagues (1994) that anti-TNF-α administered to swine made septic with *Pseudomonas aeruginosa* reduced the sequestration of PMNs into the lung. While the pathophysiological significance of the neutrophilia induced by O_3 exposure is a matter of some debate, PMNs are capable of releasing a variety of cytokines, proteases, and reactive oxygen species that could be injurious

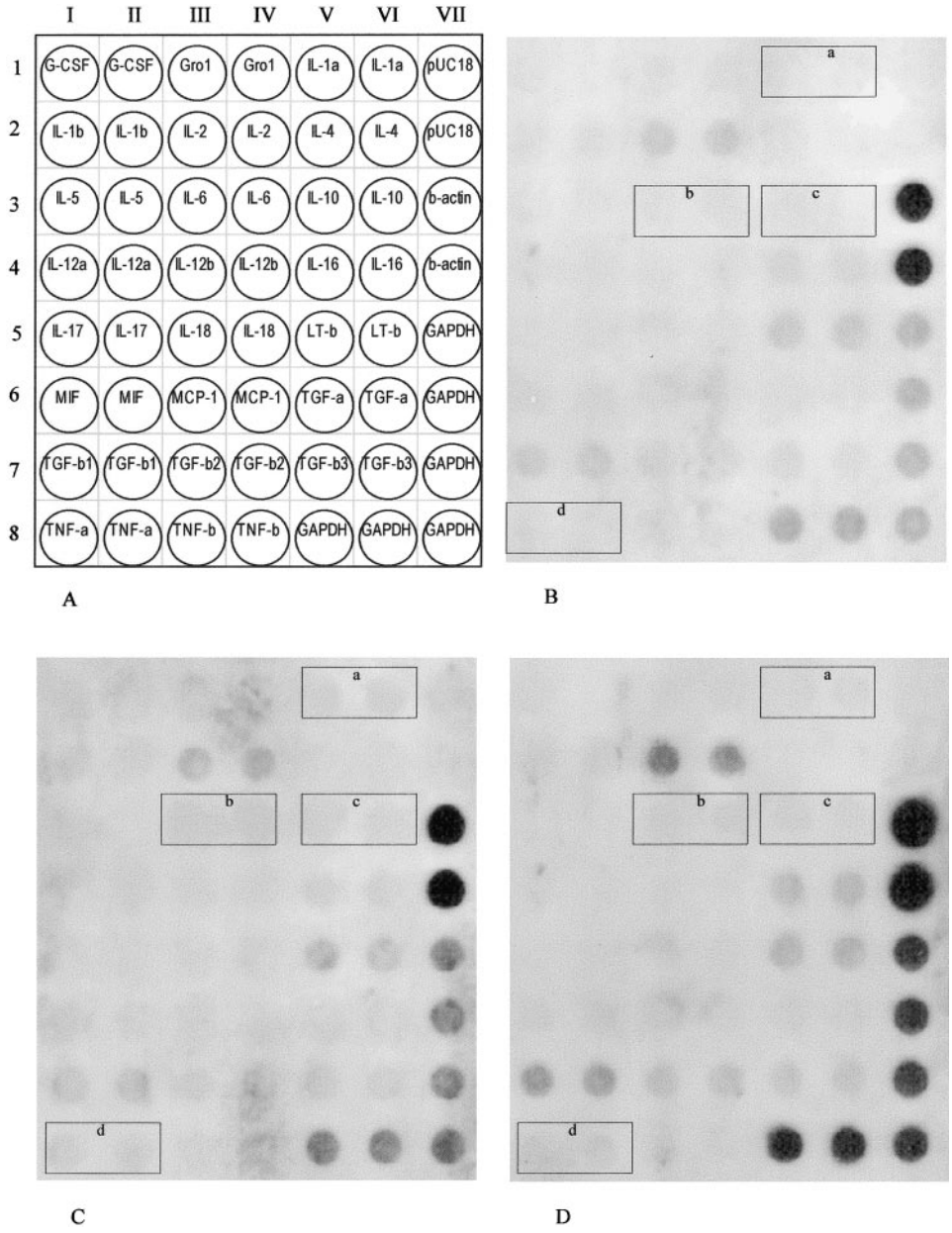

FIG. 5. Gene array template (A) displaying spot locations for cDNAs for selected cytokines, and scanned images of array blots for air-exposed controls (B), O_3 (C), and O_3 + anti-TNF-α (D) groups. Control animals received preimmune rabbit IgG prior to exposure. The blots marked by rectangles indicate genes that were differentially expressed following O_3 exposure, and downregulated by anti-TNF-α treatment prior to O_3 exposure. (a) IL-1α; (b) IL-6; (c) IL-10; (d) TNF-α.

to the epithelial cells. We have previously shown that by depleting leukocytes with cyclophosphamide, the BALF albumin levels following O_3 exposure could be significantly reduced as compared to controls (Bhalla *et al.*, 1992). In our present study the neutralization of TNF-α *in vivo* failed to completely abolish the influx of PMNs into the air spaces. This observation suggests that other chemotactic factors are involved in the recruitment of PMNs following O_3 exposure. While O_3 exposure resulted in a significant increase in PMNs, the overall BALF cell counts were similar between the groups. Since PMNs constitute only a small fraction of the total inflammatory cell population in the lung, a change in the number of PMNs does not significantly impact the total cell number. Furthermore, a decrease in macrophage number after O_3 exposure has been reported. This is attributed to the effect O_3 has on macrophage adherence (Bhalla, 1996). Greater adhesion of macrophages following O_3 exposure would promote their retention in the lung and reduced recovery by BAL.

Fibronectins are multifunctional proteins that provide a site for cell attachment, thereby promoting cell adhesion, cell migration, and wound repair (Hynes, 1990; Yamada, 1987). Enhanced fibronectin expression has been observed in a variety of lung disorders and after O_3 exposure of animals (Gupta *et al.*, 1998; Limper and Roman, 1992). A significant increase in fibronectin levels in the BALF of O_3 exposed animals over that in air-exposed controls in this study is consistent with the

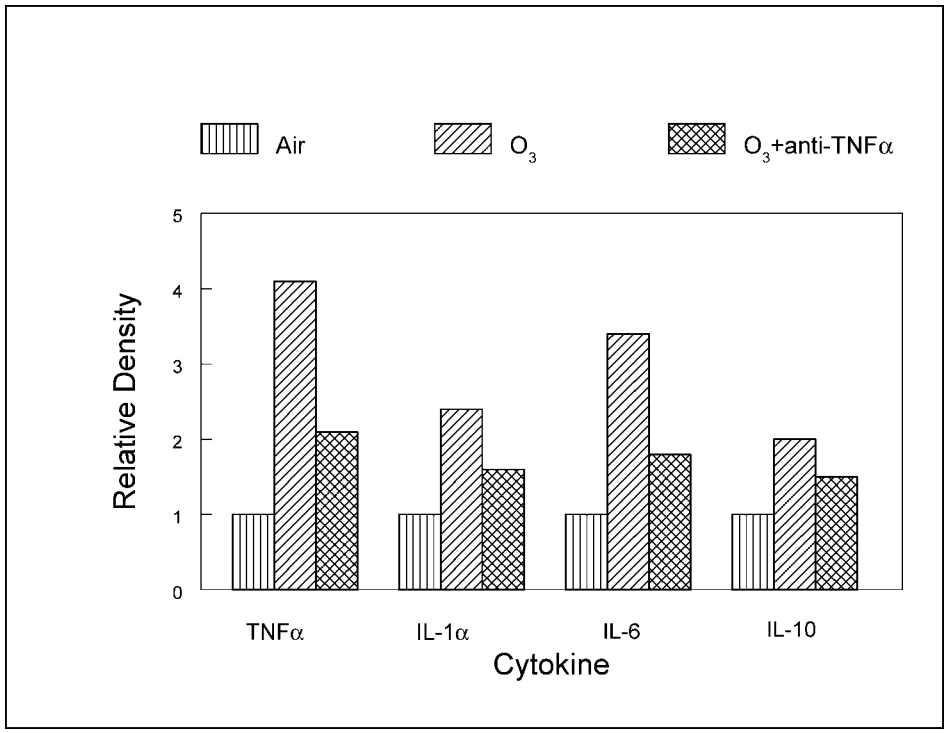

FIG. 6. Densitometric reading of cytokine gene expression in the lungs, as detected by gene array. The values are means of duplicate samples for each cytokine. Values of air-exposed controls were normalized to 1.

earlier reports. Although the role of fibronectin in injury and inflammation is supported by its ability to promote tissue repair and cell recruitment (Denholm *et al.,* 1989; Everitt *et al.,* 1996; Rennard *et al.,* 1981), in our study the treatment with anti-TNF-α had no effect on the BALF fibronectin levels despite a reduction in injury and inflammation. This suggests that fibronectin upregulation as a result of O_3 exposure is independent of the effects of TNF-α release under these experimental conditions.

Besides increased permeability and recruitment of inflammatory cells, pulmonary responses to environmental and other insults are characterized by the activation of recruited cells for the release of inflammatory cytokines. While the increase in number of PMNs provides a morphological indication of inflammation, the release of cellular mediators reflects their state of activation. The mediator release in response to O_3 has been studied both in tissue culture and in *in vivo* settings. These studies have shown increased *in situ* levels of TNF-α and IL-1α in rats infected with *Listeria monocytogenes* and exposed to O_3 (Cohen *et al.,* 2001), an increased release of TNF-α and IL-1β by macrophages isolated from rats exposed to O_3 (Ishii *et al.,* 1997), and increased release of IL-1α, IL-6, IL-8, and TNF-α following exposure of nasal mucosa to O_3 in an organ culture system (Schierhorn *et al.,* 1999). IL-1, secreted by macrophages, is also increased in infectious diseases and inflammatory processes (Le and Vilcek, 1987). Therefore, we regard an upregulation of IL-1 gene following O_3 exposure of rats as an observation consistent with its role as an inflammatory stimulus. Since IL-1 release may be regulated by TNF-α, it is not surprising that the induction of IL-1 gene by O_3 was blocked in animals treated with anti-TNF-α antibody prior to O_3 exposure. As a simple explanation, it can be assumed that the treatment with anti-TNF-α antibody caused an attenuation of inflammation through an IL-1 sensitive pathway. However, this interpretation fails to emphasize the notion that inflammatory response is the result of a complex set of events involving multiple cytokines and other cellular components and molecules. In addition to changes in IL-1 gene, our gene array approach revealed an upregulation of proinflammatory IL-6 and anti-inflammatory IL-10 following O_3 exposure. The expression of these cytokines was blocked by anti-TNF-α treatment. IL-6, a chemotactic cytokine, is implicated in the recruitment and activation of PMNs. Its role in O_3 induced lung inflammation is supported by studies demonstrating increased release of this cytokine in animals (Mango *et al.,* 1998; McKinney *et al.,* 1998) and humans (Torres *et al.,* 1997) following exposure. In humans, IL-6 release immediately postexposure correlated with the PMN peak at 18 h after exposure of adult smokers and nonsmokers to O_3. In the current study, IL-6 expression supports the role of this cytokine in O_3 induced changes in the lung.

Despite a growing interest in the role of cytokines in the induction of lung inflammation, not much is known about the effects of O_3 on the production and release of IL-10. In a human study IL-10 was detected in the BAL from 6 out of 23 subjects exposed repeatedly to 200 ppb O_3 (Jorres *et al.,* 2000). In our rats exposed to a much higher O_3 concentration, IL-10 gene was clearly upregulated. The expression of IL-10 is

contrary to the expected downregulation of this cytokine by proinflammatory stimuli, such as O_3 and TNF-α. A likely explanation for this observation is the production of proinflammatory TNF-α and IL-1α by the PMNs recruited in the lung following O_3 exposure, and an effort on the part of macrophages to downregulate these cytokines and maintain a balance through an increased production of anti-inflammatory IL-10. This explanation is supported by other injury models, where increases in IL-10 levels were observed after inflammatory insults. Steinhauser and colleagues (1999) have reported an increase in IL-10 production following *P. aeruginosa* challenge in septic mice. This increase was associated with an influx of PMNs and an increase in myeloperoxidase activity in the lung. The suggestion that IL-10 is a modulator of lethal excessive production of inflammatory cytokines (Steinhauser *et al.,* 1999), and the observation that the neutralization of IL-10 caused an excessive expression of proinflammatory cytokines and death (Howard *et al.,* 1993; Standiford *et al.,* 1995) further support its role in limiting inflammation. We, therefore, propose that the induction of this anti-inflammatory cytokine serves to regulate inflammation as a part of recovery process following initial injury.

Although the results of this study show that the treatment of animals with anti-TNF antibody caused a reduction in O_3-induced PMNs, albumin, TNF-α, IL-1α, IL-6, and IL-10, the levels were still higher than the baseline values. While these results offer an insight into the inflammatory mechanisms and provide support to the role of TNF-α as an important regulatory cytokine, they recognize a complex mechanism involving multiple cytokines. It is possible that the administration of serum along with anti-TNF-α antibody caused some inflammation and partially compromised the attenuating effects of the antibody. It is also likely that cytokines and chemokines other than those examined in this study contribute to lung inflammation. Additionally, chemokines that are not chemotactic for PMNs may be modified to recruit PMNs. For instance, C-C chemokines, such as MCP-1 and RANTES, are primarily regarded as chemoattractants for monocytes and lymphocytes, while C-X-C chemokines, such as MIP-2 and IL-8, are chemoattractants for neutrophils (Baggiolini *et al.,* 1994; Bonecchi *et al.,* 1999; Premack and Schall, 1996). Although the induction of neutrophilic inflammation in the lung is not associated with a change in MCP-1 and RANTES levels (Johnston *et al.,* 1998), PMNs activated by proinflammatory cytokines under certain conditions undergo a change in their receptor expression and become responsive to CC chemokines (Cheng *et al.,* 2001). These studies raise the possibility that stimuli such as O_3 could cause a change in receptor expression and induce responsiveness to CC chemokines. Although this possibility remains to be tested, it provides an alternative mechanism for lung inflammation and an explanation for partial reversal of O_3 effects by anti-TNF-α antibody.

In summary, we have shown that the administration of anti-TNF-α prior to O_3 exposure can significantly reduce the inflammation and epithelial injury as evidenced by the reduction in PMNs and BALF albumin levels. However, the neutralization of TNF-α did not completely abolish the O_3-induced effects, adding further support to the idea that the pathophysiology seen following O_3 exposure is a result of a variety of complex cellular and molecular interactions. Some of these interactions are evident in the cytokine genes expressed in response to O_3 exposure and anti-TNF-α treatment. Both TNF-α and IL-1 are multifactorial cytokines and may act in concert. Because IL-1 may be a stimulus for the production of IL-6 (Sugawara *et al.,* 2001), the upregulation of IL-6 gene is a likely consequence of IL-1 activation by O_3. This sequence may also provide an explanation to the role of anti-TNF-α in reducing O_3-induced inflammation. Accordingly, a modulatory role of anti-TNF-α may be exerted through an attenuation of IL-1, which in turn leads to the downregulation of IL-6 and decreased inflammation.

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