

Development of Pulmonary Tolerance in Mice Exposed to Zinc Oxide Fumes

Scott C. Wesselkamper, Lung Chi Chen, and Terry Gordon¹

Department of Environmental Medicine, New York University School of Medicine, 57 Old Forge Road, Tuxedo, New York 10987

Received August 23, 2000; accepted October 13, 2000

As a result of repeated exposures to inhaled toxicants such as zinc oxide (ZnO), numerous individuals acquire tolerance to the exposures and display reduced symptoms. To ascertain whether tolerance is developed in an animal model, NIH-Swiss mice were exposed to 1.0 mg/m³ ZnO for 1, 3, or 5 days (1X, 3X, or 5X), and polymorphonuclear leukocyte (PMN) and protein levels in bronchoalveolar lavage (BAL) were measured. Mice acquired tolerance to neutrophil infiltration into the lungs, as total PMNs returned near baseline in 5X-exposed animals as compared to that of the 1X exposure group (1X = $2.7 \pm 0.4 \times 10^4$, 5X = $0.2 \pm 0.1 \times 10^4$, mean \pm SE, $p < 0.05$). Development of tolerance to changes in lavageable protein, however, was not observed (1X = 313 ± 29 μ g/ml, 5X = 684 ± 71 μ g/ml, $p < 0.05$). Tolerance to PMN influx did not persist following re-exposure to ZnO after 5 days of rest. In contrast to ZnO exposure, following single and repeated exposure to aerosolized endotoxin there was development of tolerance to protein in BAL (1X = 174 ± 71 μ g/ml, 5X = 166 ± 14 μ g/ml, $p > 0.05$), but not to PMN influx (1X = $5.5 \pm 1.7 \times 10^4$, 13.9 $\pm 1.7 \times 10^4$, $p < 0.05$). Induction of lung metallothionein (MT) was also observed in mice exposed once or repeatedly exposed to ZnO, suggesting that MT may play a role in its molecular mechanism.

Key Words: zinc oxide; tolerance; metallothionein; metal fume fever; endotoxin; adaptation.

Workers are exposed daily to a variety of inhaled toxic agents in many different occupational settings. Individuals can be exposed to zinc oxide (ZnO) fumes that are generated from welding processes (Dryson and Rogers, 1991), or bacterial endotoxin (lipopolysaccharide) associated with cotton dust and contaminated humidifiers (Donham, 1986; Flaherty *et al.*, 1984). Inhalation exposure to these agents has been shown to induce diverse pulmonary effects in laboratory animals and human subjects, including inflammatory cellular influx of polymorphonuclear leukocytes (PMNs) and dyspnea (Gordon *et al.*, 1991, 1992; Kuschner *et al.*, 1997). As a result of frequent and repeated exposures to such inhaled toxicants, individuals may develop tolerance to the exposures and exhibit reduced symptoms. Tolerance can be defined as the ability to resist the adverse actions of a toxicant following repeated exposures.

¹ To whom correspondence should be addressed. Fax: (845) 351-5472. E-mail: gordont@env.med.nyu.edu.

The implications of tolerance to health risks in humans are numerous, having both advantages and disadvantages with respect to the development of detrimental health effects. In the present study, ZnO is used as the model agent to begin investigating the pathogenesis and underlying mechanisms of pulmonary tolerance. Exposure to ZnO has been shown to produce a number of adverse health effects in humans, including metal fume fever (MFF), an illness characterized mainly by pulmonary inflammation, chills, fever, malaise, and myalgia (Blanc *et al.*, 1991; Fine *et al.*, 1997; Sturgis *et al.*, 1927).

Drinker and associates (1927) were the first to document the development of pulmonary tolerance to repeated ZnO exposures. In a recent investigation of MFF in zinc foundry workers, acquisition of tolerance from repeated ZnO exposure could potentially explain the absence of MFF cases observed in the study (Martin *et al.*, 1999). Similar to the systemic effects of ZnO, repeated exposure to airborne endotoxin/cotton dust produces a clinical response known as "Monday morning fever," a condition where workers develop tolerance to acute symptoms during the course of a work week, but have a worsening of symptoms after a weekend hiatus with no significant exposure (Rose and Blanc, 1998; Rylander, 1990). Unlike ZnO, however, tolerance to endotoxin/cotton dust inhalation may manifest later as lasting respiratory problems such as byssinosis (Christiani *et al.*, 1999).

Little is actually known about the molecular mechanism of tolerance to inhaled toxicants. One possible means by which tolerance to ZnO exposure may occur entails the induction of metallothionein (MT) genes by zinc. Metallothioneins are low-molecular-weight proteins that bind strongly to metals (Klaassen and Lehman-McKeeman, 1989; Palmiter, 1998). Induction of MT genes from zinc exposure is a common cellular response in most animal and human tissues (Sadhu and Gedamu, 1988). It has been hypothesized that the induction of MT may serve as an adaptive mechanism that diminishes the extent of metal-induced toxicological injury (Klaassen and Liu, 1998). It is believed that MT protein protects cells from metal toxicity via sequestration mechanisms and scavenging of free radical species (Sato and Bremner, 1993; Thornalley and Vasak, 1985). In previous work, we demonstrated an approximate 8-fold elevation over control in MT gene expression in the lungs of rats

exposed to 2.5 mg/m^3 ZnO for 3 h, as well as a 3.5-fold elevation after exposure to 1.0 mg/m^3 ZnO (Cosma *et al.*, 1992). Cytokines and glucocorticoids have also been shown to induce synthesis of metallothionein proteins (Liu *et al.*, 1991; Schroeder and Cousins, 1990). MT may therefore be responsible, in part, for maintaining physiologic levels of essential metals such as zinc and may have a role in eliminating excessive amounts of metals introduced by such means as inhalation of metal fumes. Thus, MT may play a role in the development of tolerance to inhaled toxic metals.

A human model of tolerance is rather impractical and has limited utility in answering questions about potential mechanisms. Therefore, the purposes of this study were twofold: (1) to generate an animal model to study the development of pulmonary tolerance following successive exposures to ZnO, and (2) to address the hypothesis that induction of MT is associated with the development of pulmonary tolerance. Pulmonary responses in outbred mice exposed once or repeatedly to ZnO fume were examined by measuring PMN and protein concentrations in BAL, pulmonary pathology, and induction of MT gene expression. The development of pulmonary tolerance was also assessed after single and repeated exposures of mice to aerosolized endotoxin to determine whether the acquisition of pulmonary tolerance was agent-specific.

MATERIALS AND METHODS

Animals. Male NIH-Swiss mice (7 to 9 wk, 29–36 g) were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). All mice were acclimated for at least 1 wk before exposure and housed in a positive pressure environment with a 12-h light/dark cycle starting at 6:00 A.M. All mice were provided with water and standard laboratory rodent chow (Purina, Indianapolis, IN) *ad libitum*, except during exposure.

Experimental design. To evaluate pulmonary tolerance, mice were exposed to ZnO fumes on one day (1X, $n = 7$), on 3 successive days (3X, $n = 5$), or on 5 successive days (5X, $n = 5$) for 3 h/day. Mice ($n = 5$) were also exposed to air for control purposes. All 3X exposures were done concurrently with 5X exposures, and all 1X exposures were done on the same day as the final 5X exposure. Additionally, mice were exposed to aerosolized endotoxin 1X ($n = 4$) or 5X ($n = 4$) or saline (control, $n = 6$) for 3 h/day. Acquisition of pulmonary tolerance was assessed by measuring mean total PMN and protein concentrations in BAL fluid as well as lung pathology 24 h postexposure (1X versus 3X and/or 5X exposures). Induction of MT was evaluated at 0 h postexposure in mice exposed to ZnO fumes 1X, 3X, or 5X or air ($n = 4$ /exposure group). To determine whether pulmonary tolerance to inhaled ZnO is persistent, mice were exposed for 3 h/day on 5 consecutive days, allowed to rest for 5 days, and then re-exposed to ZnO (ZnO/rest/ZnO, $n = 5$) or air (ZnO/rest/air, $n = 5$).

Atmosphere generation and characterization. Mice were exposed to 1.0 mg/m^3 ZnO in stainless steel cages placed inside a 0.07 m^3 plexiglass chamber. The exposure level of ZnO used in this study was one-fifth of the current threshold limit value (TLV) for an 8-h workday and 40-h workweek (ACGIH, 1991). ZnO fume was generated in a modified furnace system based on a previous design (McCarthy *et al.*, 1982). Briefly, 1.7 g of granular zinc metal (30 mesh; Fisher Scientific, Fair Lawn, NJ) were placed in a ceramic crucible and heated to approximately 550°C in a furnace. Zinc vapor was carried downstream through the furnace by inert, high purity argon gas to react with oxygen yielding a supersaturated atmosphere of ZnO vapor. This vapor condensed to yield ultrafine particles of ZnO, which were mixed with filtered air

in a series of cooling, humidifying, and diluting zones before entering the exposure chamber. These primary particles aggregate in chains to form secondary particles (McCarthy *et al.*, 1982). Measurements performed with a differential mobility analyzer (Model 3021, TSI, St. Paul, MN) demonstrated that these secondary particles have a mass median aerodynamic diameter (MMAD) of $0.3 \mu\text{m}$ and geometric standard deviation (σ_g) of 1.5. The ZnO fume was diluted with metered room air filtered through an activated charcoal filter and a HEPA filter and humidified to 30–50% relative humidity. During exposures, samples of the chamber atmosphere were collected approximately every 30 min from the manifold of the exposure system with polytetrafluoroethylene filters (Type TX40HI20-WW, Pallflex Products Corp., Putnam, CT). Each filter was weighed on a microbalance (Model C-30, Cahn Instruments, Cerritos, CA) and ZnO concentration was determined gravimetrically. Based on each filter weight, the furnace system was immediately adjusted to yield the desired output of ZnO. The control (sham) exposures consisted of furnace gas composed of 97% filtered room air and 3% argon.

For endotoxin exposures, mice were placed in stainless steel cages inside a 0.05 m^3 plexiglass chamber. Endotoxin and saline (control) aerosols were generated with a Babington-type nebulizer (Solosphere, Airlife, Inc., Modesto, CA) driven by HEPA-filtered air at 9 psi. The output of the nebulizer was diluted with HEPA-filtered air at 15 l/min prior to entering the exposure chamber. A solution of endotoxin ($3.5 \mu\text{g/ml}$ of lipopolysaccharide; E. coli 0127:B8, Difco Laboratories, Detroit, MI) was made up with pyrogen-free, isotonic saline (0.9% sodium chloride, Baxter Healthcare Corp., Deerfield, IL) immediately prior to exposure. The concentration of endotoxin used in these exposures was chosen from previous animal studies in this laboratory (Gordon *et al.*, 1991; Hur *et al.*, 1999). The nebulizer reservoir was emptied and refilled at 1-h intervals during exposures to minimize concentrating effects of the nebulizer. Exposure concentrations were determined by sampling the chamber atmosphere in the breathing zone of the animals twice during each exposure, and analyzing endotoxin concentrations with a Limulus amoebocyte lysate assay (QC1000, Whittaker Bioproducts, Walkersville, MD) using a spectrophotometric microplate method (Gordon, 1992). The MMAD of saline and endotoxin particles produced by the nebulizer under the specified exposure conditions was $1.9 \mu\text{m}$ with σ_g of 2.2 as determined with a Mercer impactor (Intox Products, Albuquerque, NM).

Bronchoalveolar lavage (BAL). At 24 h postexposure to ZnO, endotoxin, air, or saline, mice were killed by intraperitoneal injections of ketamine HCl (100 mg/kg ; Vetalar, Fort Dodge Laboratories, Fort Dodge, IA) and sodium pentobarbital (175 mg/kg ; Sleepaway, Fort Dodge Laboratories), and the posterior abdominal aorta was severed. The lungs of each mouse were lavaged 2 times with 1.2 ml of Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (pH 7.2–7.4, 37°C ; GibcoBRL, Life Technologies, Grand Island, NY). Recovered BAL fluid was immediately placed on ice (4°C). Aliquots ($100 \mu\text{l}$) of lavage fluid were cytocentrifuged (Cytospin, Shandon Southern Products Ltd., UK), and the cells were stained with Hemacolor (EM Science, Gibbstown, NJ) for differential cell analysis. Differential cell counts were performed by identifying at least 200 cells according to standard cytological procedures (Saltini *et al.*, 1984). Total cell counts were determined with a hemacytometer. The lavage fluid was then centrifuged (500 g , 8 min, 4°C) and the supernatant was decanted. The total protein concentration in the supernatant was measured using an assay kit that follows the method of Bradford (1976) and is accurate from 10 to $2,000 \mu\text{g/ml}$ (bovine serum albumin standard, 550 nm; Pierce, Rockford, IL). Total protein concentration in BAL fluid was used as an indicator of changes in lung permeability and injury.

Tissue preparation and lung pathology. To obtain lung tissue for RNA isolation, the chest cavity was opened and the lungs were excised, flash-frozen in liquid nitrogen, and stored at -70°C until RNA extractions were performed. To obtain tissue for pathological analysis, a cannula (0.6 mm internal diameter) was inserted into the trachea and the lungs were instilled in situ with 2% glutaraldehyde, removed, and immersed in fixative. Fixed tissues were trimmed, dehydrated through a series of ethanol solutions, and processed into paraffin blocks (Hypercenter XP, Shandon Scientific). Paraffin-embedded tissues were sectioned ($5 \mu\text{m}$) and stained with hematoxylin and eosin.

RNA isolation and analysis. Total cellular RNA was isolated from lung tissue with TRIzol (Life Technologies). RNA quantity and purity was determined by A260/A280 spectrophotometric absorbances. In addition, integrity of RNA was confirmed by ethidium bromide staining of ribosomal RNA following gel electrophoresis. Standard slot-blot RNA analyses were carried out by direct application of 10 μ g glyoxalated RNA samples onto nylon membrane filters (Nytran, Schleicher & Schuell, Keene, NH) in a vacuum manifold (Schleicher & Schuell). Membrane-bound RNA was hybridized to nick-translated, 32 P-labeled cDNA probes in the presence of dextran sulfate by a modification of the procedure of Wahl *et al.* (1979). The MT-1 plasmid was provided by Dr. H. Herschman (UCLA), and the β -actin was commercially purchased (ATCC, Bethesda, MD). Following hybridization, filters were washed to a final stringency of $0.4 \times$ standard saline citrate + 0.1% sodium dodecyl sulfate at 65°C for 30 min. Specifically hybridized mRNA was visualized by film autoradiography at -70°C using Kodak XAR-5 film plus Cronex intensifying screens (DuPont, Wilmington, DE). Autoradiogram signal strengths of hybridized mRNA were quantitated by the measurement of optical densities. MT-1 gene expression results were normalized to β -actin expression, serving as an internal control to ensure that artifacts such as unequal loading of RNA onto filters were not responsible for any observed differences in the strengths of autoradiographic signals. Due to possible variability in baseline values of gene expression over time and between batches of animals, control mice were also exposed to air (sham) and analyzed.

Data analysis. To evaluate ZnO- and endotoxin-induced changes in PMNs and protein in BAL fluid at various time points, all values are presented as the mean \pm SE, and differences between means were assessed using a one-factor analysis of variance (ANOVA) followed by a Student-Newman-Keuls (SNK) multiple-comparison test of significance. MT-1 mRNA levels normalized to β -actin are presented as the mean \pm SE, and statistical analysis was performed with a one-factor ANOVA and SNK test of significance. Statistical significance for all comparisons was accepted at $p < 0.05$.

RESULTS

PMN and protein concentrations in ZnO- and endotoxin-exposed mice. Compared to air-exposed control animals, both 1X and 3X ZnO-exposed mice had significant increases in the percentage of PMNs recovered in BAL fluid. The 1X ZnO-exposed mice also had significantly greater total PMNs (Figs. 1A and 1B). Compared to the 1X exposure group, both 3X and 5X ZnO-exposed mice had significantly lower total and percent PMNs in BAL fluid (Figs. 1A and B). In contrast to the repeated ZnO exposure-related decrease in PMNs, 1X, 3X, and 5X ZnO-exposed mice had significantly greater levels of protein in BAL fluid compared to air-exposed controls, and the 3X and 5X ZnO exposure groups had significantly greater protein levels compared to 1X-exposed mice (Fig. 1C).

Unlike ZnO-exposed animals, mice exposed to endotoxin exhibited little evidence of pulmonary tolerance. Following repeated endotoxin exposure, there was a significant increase in total and percent PMNs in the BAL fluid from 5X-exposed mice as compared to that of 1X-exposed mice. Total and percent PMNs were also significantly increased over saline-exposed controls in both the 1X and 5X exposure groups (Figs. 2A and 2B). In contrast to ZnO exposure, total protein concentrations were not significantly affected by single or repeated endotoxin exposures when compared to saline-exposed controls (Fig. 2C).

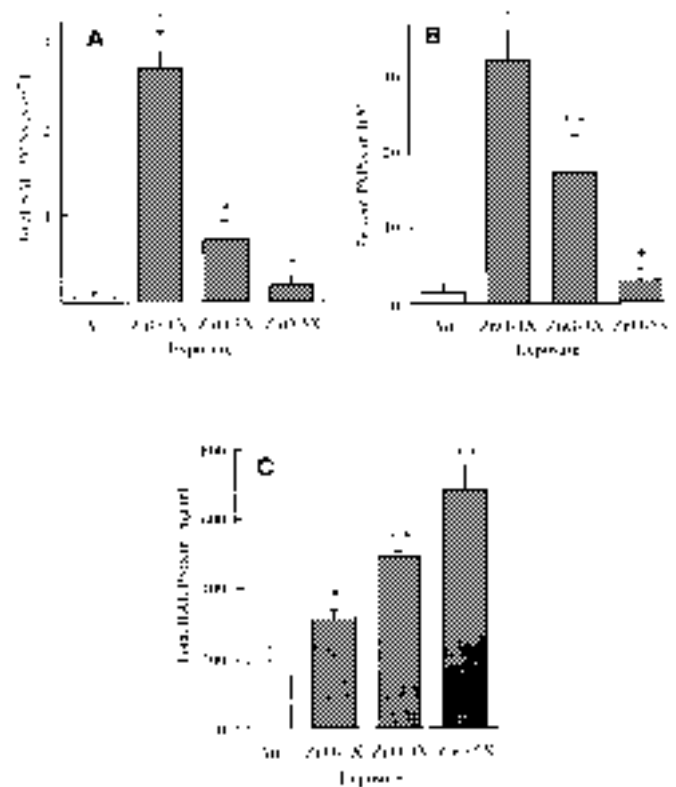


FIG. 1. Total number of polymorphonuclear leukocytes (PMNs) (A), percent PMNs (B), and total protein (C) in BAL fluid of NIH-Swiss mice 24 h postexposure to 1.0 mg/m³ ZnO 1X ($n = 7$), 3X ($n = 5$), or 5X ($n = 5$) or air (control, $n = 5$) for 3 h. Values are means \pm SE; * denotes significant difference from air-exposed controls; $p < 0.05$. + denotes significant difference from 1X exposure group; $p < 0.05$.

Pulmonary histopathology from repeated ZnO exposures. When compared to those of air-exposed controls (Fig. 3A), lung sections of 1X-exposed mice had no discernible pathological differences (Fig. 3B). When qualitatively compared to both air and 1X exposure groups, however, lung sections of 5X exposed animals appeared to have alveolar septal wall thickening, cellular congestion and infiltrates in the air spaces, greater perivascular distention, and focal loss of epithelial integrity (Fig. 3C). These observations are consistent with the aforementioned BAL protein changes following 1X and 5X ZnO exposures.

Persistence of pulmonary tolerance to ZnO. The results of investigating the persistence of tolerance are presented in Figure 4. Percent and total PMNs as well as total protein in BAL fluid of the ZnO/rest/ZnO exposure group did not differ from 1X-exposed animals, suggesting that pulmonary tolerance to ZnO did not persist following a 5-day rest period. ZnO/rest/ZnO-exposed animals had significantly greater levels of all cellular and biochemical parameters as compared to that of the control ZnO/rest/air exposure group. ZnO/rest/air-exposed mice had significantly lower percentages and total PMNs in BAL fluid (but not total protein) compared to 1X-exposed

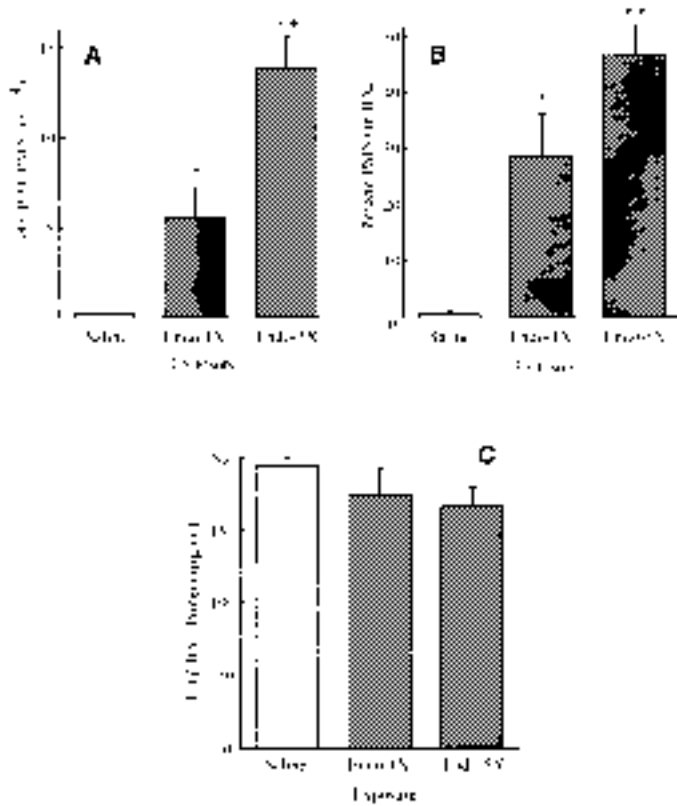


FIG. 2. Total number of polymorphonuclear leukocytes (PMNs) (A), percent PMNs (B), and total protein (C) in BAL fluid of NIH-Swiss mice 24 h postexposure to aerosolized endotoxin (generated from a 3.5 $\mu\text{g}/\text{ml}$ solution) 1X ($n = 4$) or 5X ($n = 4$) or saline (control, $n = 6$) for 3 h. Values are means \pm SE; * denotes significant difference from saline-exposed controls; $p < 0.05$; + denotes significant difference from 1X exposure group; $p < 0.05$.

animals (Fig. 4), but did not significantly differ from the air (control) exposure group in any BAL fluid measurement.

Lung metallothionein mRNA induction. Exposure to 1.0 mg/m^3 ZnO induced a sustained and significant increase in lung MT-1 gene expression over the 5-day exposure period. An 8-fold elevation over control in MT-1 mRNA was observed after the 1X exposure, and approximate 6-fold elevations were seen following the 3X and 5X exposures (Fig. 5). The relative levels of MT-1 mRNA were not significantly different among the 1X, 3X, and 5X ZnO exposure groups.

DISCUSSION

In previous human and animal studies, the magnitude of lung inflammation (as measured by PMNs in BAL) and increased lung permeability and injury (as measured by total protein in BAL) have been used as indices to assess the development of pulmonary tolerance following repeated exposure to a variety of inhaled toxicants. For example, the development of pulmonary tolerance from endotoxin exposure is associated with a significantly diminished PMN response,

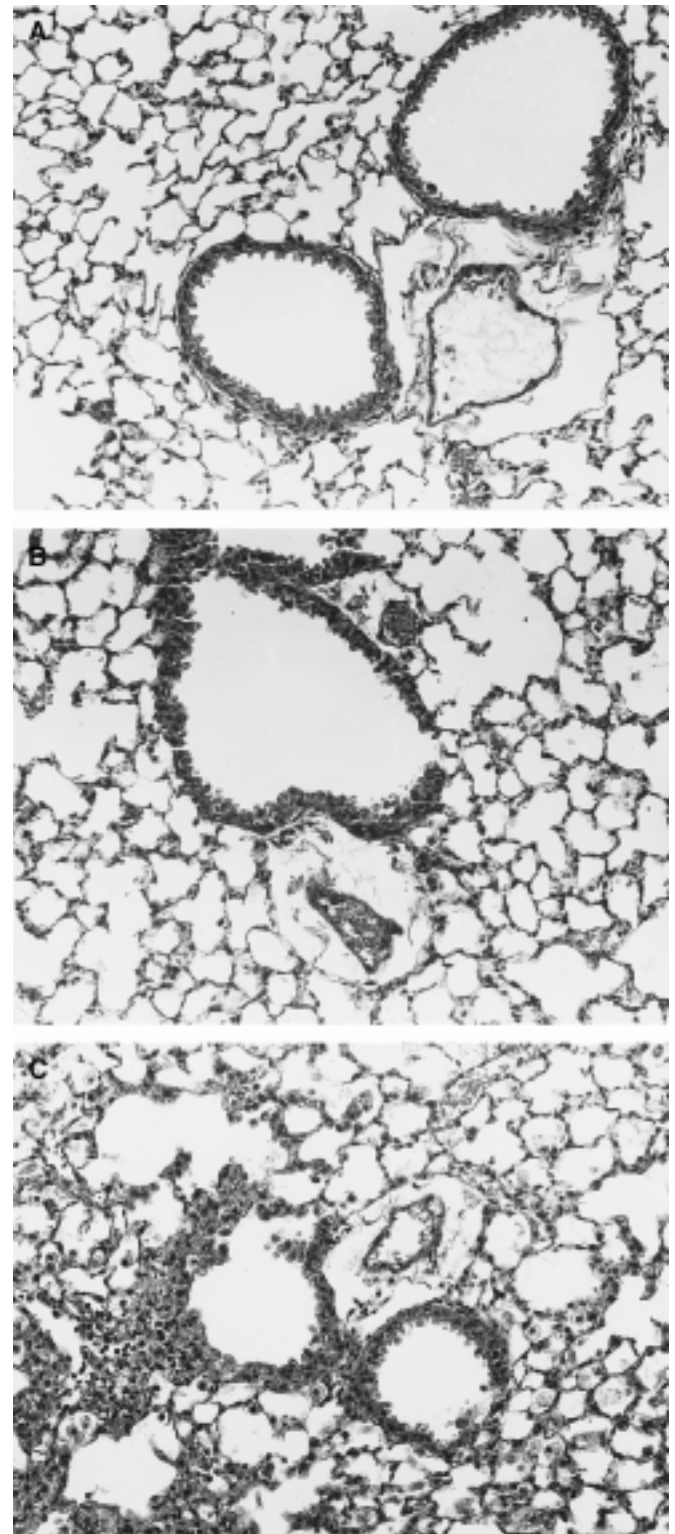


FIG. 3. Lung histology of NIH-Swiss mice 24 h postexposure to 1.0 mg/m^3 ZnO 1X or 5X or air (control) for 3 h. Tissues were fixed with 2% glutaraldehyde, embedded in paraffin, stained with hematoxylin and eosin, and viewed by light microscopy. Original magnification $\times 200$. (A) air-exposed control. (B) 1X ZnO-exposed. (C) 5X ZnO-exposed.

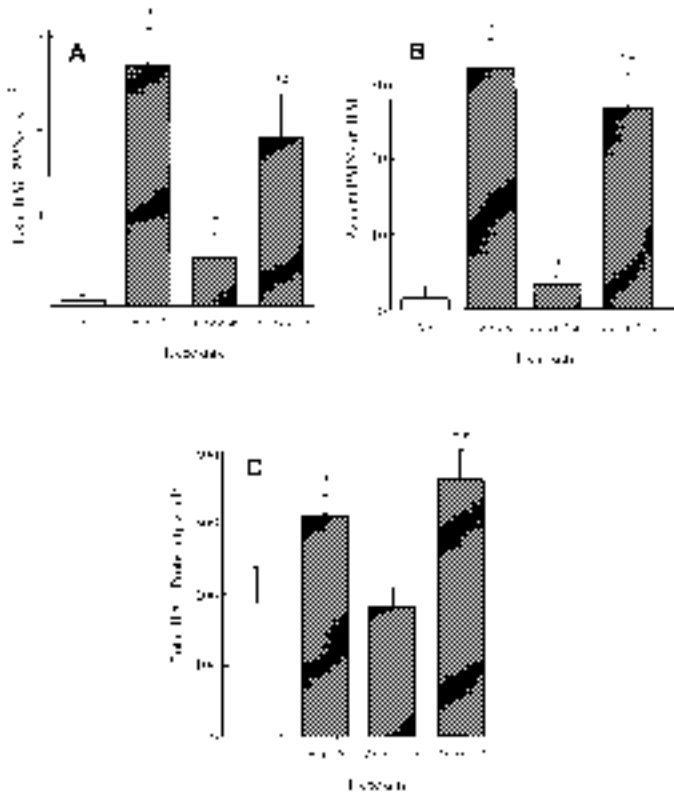


FIG. 4. Total number of polymorphonuclear leukocytes (PMNs) (A), percent PMNs (B), and total protein (C) in BAL fluid of NIH-Swiss mice 24 h postexposure to 1.0 mg/m³ ZnO 1X ($n = 7$), Zn/rest/Zn ($n = 5$), or Zn/rest/air ($n = 5$) or air ($n = 5$) for 3 h. Zn/rest/Zn and Zn/rest/air animals were exposed to ZnO for 5 days, allowed to rest for 5 days, then rechallenged with ZnO or air, respectively, for 3 h. * Denotes significant difference from air-exposed controls; $p < 0.05$; + denotes significant difference from 1X exposure group, $p < 0.05$; # denotes significant difference from Zn/rest/air exposure group; $p < 0.05$.

while lung permeability remains unaltered (Schwartz *et al.*, 1994; Shimada *et al.*, 2000). In the development of ozone tolerance, however, protein levels in BAL remain elevated after repeated exposures while PMN values decreased, suggesting that lung injury continues to progress despite attenuation of inflammatory cellular influx (Christian *et al.*, 1998; Devlin *et al.*, 1997; Jörres *et al.*, 2000; Tepper *et al.*, 1989).

In the present study, we have demonstrated that after repeated exposure of outbred mice to ZnO, total and percent PMNs in BAL were significantly decreased after 3 exposures, and continued decreasing to near baseline following 5 exposures. Thus, these mice developed tolerance to the invasion of neutrophils into the lungs after consecutive ZnO exposures. Total protein levels in BAL fluid, however, continued to increase significantly over successive exposures. This suggests that, despite the tolerance to neutrophil influx, mice did not become tolerant to ZnO-induced changes in lung permeability and injury. This was also verified in the greater lung pathology of mice exposed repeatedly to ZnO compared to those exposed

only one time. Similar to the aforementioned investigations with ozone, our data suggest that the development of tolerance to ZnO-induced PMN infiltration into the lungs may not necessarily be beneficial because lung injury continued to progress over the 5-day exposure period.

In contrast to mice exposed repeatedly to ZnO, mice exposed to aerosolized endotoxin for 5 successive days did not become tolerant to the influx of neutrophils. These 5X-exposed mice exhibited a 2.5-fold increase in total PMNs as compared to those exposed only one time, as well as a 70-fold increase over control values. Total protein levels in BAL fluid of endotoxin-exposed mice were not significantly increased following 1X or 5X exposures. Our single exposure data are consistent with previous findings of no changes in BAL protein despite PMN influx following a single exposure to intratracheally instilled or aerosolized endotoxin (Hudson *et al.*, 1977; Wiener-Kronish *et al.*, 1991). This lack of change in protein appears to be dose-dependent; some investigators, including ourselves (Gordon, 1992), have reported exposure to high endotoxin concentrations caused increased BAL protein in rats and guinea pigs (Li *et al.*, 1995; O'Leary *et al.*, 1996). Despite lack of evidence of tolerance to neutrophil influx from repeated endotoxin exposure in the present study, there is evidence that endotoxin produces tolerance in other test systems (Elder *et al.*, 2000; Schwartz *et al.*, 1994; Shimada *et al.*, 2000). Thus, differences in BAL data from single and repeated endotoxin exposures could be explained by the dose and route of exposure of endotoxin used in these studies. When taken together, our findings on the development of pulmonary tolerance in mice following repeated ZnO and endotoxin exposure suggest the following:

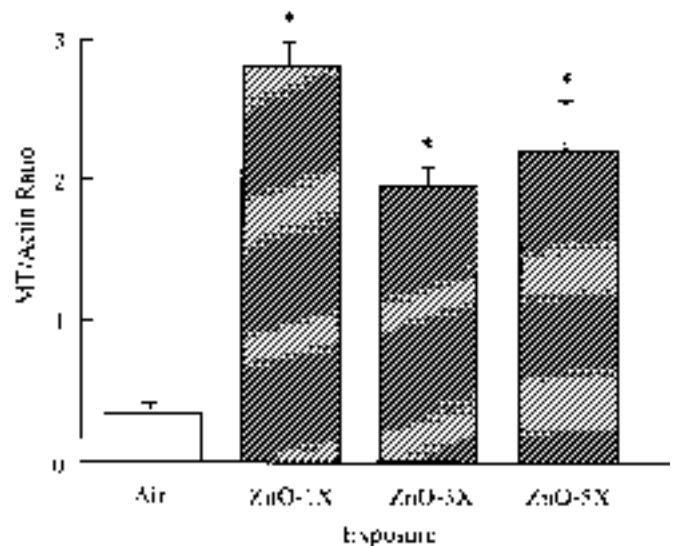


FIG. 5. MT-1 gene expression in lungs of NIH-Swiss mice 0 h postexposure to 1.0 mg/m³ ZnO 1X, 3X, or 5X or air (control) for 3h. Values were determined relative to β -actin mRNA and are presented as means \pm SE ($n = 4$ mice/exposure group); * denotes significant difference from air-exposed controls, $p < 0.05$.

- PMN influx and changes in epithelial permeability (as measured by protein in BAL) may not be mutually dependent events, which is in agreement with previous reports using other inhaled agents (Kleeberger and Hudak, 1992; Laughlin *et al.*, 1986; Raj *et al.*, 1985; Reinhart *et al.*, 1998).
- The development of pulmonary tolerance to ZnO and endotoxin may occur through different molecular mechanisms.

We also determined that pulmonary tolerance to inhaled ZnO does not persist after a 5-day rest period. Total and percent PMNs and total protein in ZnO-exposed mice, re-challenged with a single exposure to ZnO after 5 days of rest, were not significantly different from 1X-exposed mice. This is consistent with previous findings from human studies and workplace observations of "Monday morning fever" (Drinker *et al.*, 1927; Turner and Thomason, 1926). Because tolerance does not appear to be persistent, individuals who work staggered shifts, return from extended vacation, or experience high absenteeism may be at greater risk of developing adverse pulmonary or systemic effects from ZnO exposure.

Exposure to 1.0 mg/m³ ZnO induced a sustained and significant increase in MT-1 mRNA over control values following 1X, 3X, and 5X exposures. Tolerance to PMN influx into the lungs was acquired following 3X and 5X exposures. While previous investigations have shown that MT expression is altered by inflammatory cytokines such as TNF- α (Sato and Sasaki, 1992), IL-1 (Karin *et al.*, 1985; Kikuchi *et al.*, 1993), and IL-6 (Schroeder and Cousins, 1990), the role of MT in regulating the inflammatory response during the 5-day ZnO exposure period in the present study is unclear. Despite the fact that increased MT-1 expression was sustained, mice did not develop tolerance to lung injury as measured by lung pathology and total BAL protein, as these indices of injury continued to increase during the 5-day exposure period. In many cases, the induction of MT protects tissues against toxicant-induced injury (Klaassen and Liu, 1998; Waalkes and Goering, 1990). Our data suggest, however, that MT-1 induction is not associated with protection from increased lung epithelial permeability after repeated ZnO exposure. These data are consistent with independent reports of elevated lung MT gene expression following hyperoxic exposure in C57BL/6J inbred mice (Piedboeuf *et al.*, 1994), a mouse strain that is highly susceptible to hyperoxia-induced increases in lung permeability (Hudak *et al.*, 1993). Although MT has been shown to be induced by single endotoxin exposures in both rats and mice (De *et al.*, 1990; Liu *et al.*, 1991; Min *et al.*, 1991), the induction of MT-1, following repeated endotoxin exposure in the present study, was not evaluated, and further investigation is needed to elucidate its role in endotoxin tolerance.

The limitations of our animal model of tolerance to ZnO should be kept in view. Previous investigators have hypothesized that the pathogenesis of MFF in humans from ZnO

exposure is mediated by an increase in neutrophil chemoattractants and PMNs in the lung following ZnO exposure (Blanc *et al.*, 1993; Kuschner *et al.*, 1997). Although it is not known whether the development of tolerance to ZnO is paralleled by a reduction in the pulmonary inflammatory response in humans, the significant decrease in lavageable PMNs that we observed following repeated exposure of mice to ZnO provides insight into the potential role of an attenuated inflammatory response in the development of tolerance to the recurring symptoms of MFF. With regard to tolerance to lung injury as assessed by protein in BAL, changes in lavageable protein after single ZnO exposures at concentrations above the TLV have not been previously demonstrated in humans, and detrimental pulmonary effects from ZnO inhalation are currently believed to be self-limiting and without sequelae. Although the BAL protein data in our model suggest otherwise, lavageable protein has not been measured following repeated human exposures to ZnO to assess cumulative lung injury. Furthermore, our data are consistent with a previous finding in guinea pigs, which were shown to be nontolerant to increased BAL protein after multiple ZnO exposures (Conner *et al.*, 1988). We believe that the long-term and additive pulmonary effects of repeated ZnO exposures have not been thoroughly studied, as ZnO-containing welding fumes have not only been linked to transient cases of MFF, but have also been associated with more progressive adverse pulmonary effects (Ameille *et al.*, 1992; Bradshaw *et al.*, 1998). Finally, although we have proposed MT as a candidate gene in the development of pulmonary tolerance to ZnO-induced PMN influx, it should be noted that other molecular mechanisms may likewise be important. The decreased expression of proinflammatory cytokines (Flohé *et al.*, 1999), as well as the induction of anti-inflammatory cytokines (McKinney *et al.*, 1998) and antioxidants (Folz *et al.*, 1999; Wiester *et al.*, 2000) have been associated with the development of tolerance to other toxicants, and may also play a role in the mediation of tolerance to ZnO-induced PMN influx in our model.

In summary, little is known about the pathogenesis of tolerance to inhaled toxicants, and the literature is limited regarding the genes that control this response. In this study, we have demonstrated that pulmonary tolerance to PMN influx, but not BAL protein and lung pathology, is acquired in an outbred mouse model following repeated exposure to ZnO. Once developed, tolerance does not persist following 5 days of rest prior to another exposure to ZnO. MT-1 mRNA is significantly increased following 1, 3, and 5 days of ZnO exposure, but the role of MT in the development of tolerance to ZnO exposure is uncertain, as the induction of MT-1 did not confer tolerance to cumulative lung injury from repeated ZnO exposures. Future studies will seek to identify and characterize additional genes that may control the development of tolerance to inhaled particles and gases.

ACKNOWLEDGMENTS

The authors thank Margaret Krasinski, Karen Galdanes, and Diane Currie for technical assistance, and Dr. Jonathan Fine, Dr. Greg Cosma, and Dr. Stuart Horowitz for helpful advice. This study was supported by National Institute of Environmental Health Sciences Grant ES-0256 and National Institute of Occupational Safety and Health Grant RO1-OH-02987. S.C.W. was the recipient of a 2000 NCER/U.S. EPA STAR Fellowship for Environmental Graduate Study.

REFERENCES

- ACGIH (1991). *Documentation of the Threshold Limit Values and Biological Exposure Indices*, 6th ed., Vol. III, pp. 1754–1757. American Conference of Governmental Industrial Hygienists, Cincinnati.
- Ameille, J., Brechot, J. M., Brochard, P., Capron, F., and Dore, M. F. (1992). Occupational hypersensitivity pneumonitis in a smelter exposed to zinc fumes. *Chest* **101**, 862–863.
- Blanc, P. D., Boushey, H. A., Wong, H., Wintermeyer, S. F., and Bernstein, M. S. (1993). Cytokines in metal fume fever. *Am. Rev. Respir. Dis.* **147**, 134–138.
- Blanc, P., Wong, H., Bernstein, M. S., and Boushey, H. A. (1991). An experimental human model of metal fume fever. *Ann. Intern. Med.* **114**, 930–936.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein, using the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Bradshaw, L. M., Fishwick, D., Slater, T., and Pearce, N. (1998). Chronic bronchitis, work related respiratory symptoms, and pulmonary function in welders in New Zealand. *Occup. Environ. Med.* **55**, 150–154.
- Christian, D. L., Chen, L. L., Scannell, C. H., Ferrando, R. E., Welch, B. S., and Balmes, J. R. (1998). Ozone-induced inflammation is attenuated with multiday exposure. *Am. J. Respir. Crit. Care Med.* **158**, 532–537.
- Christiani, D. C., Ye, T.-T., Zhang, S., Wegman, D. H., Eisen, E. A., Ryan, L. A., Olenchock, S. A., Pothier, L., and Dai, H.-L. (1999). Cotton dust and endotoxin exposure and long-term decline in lung function: Results of a longitudinal study. *Am. J. Ind. Med.* **35**, 321–331.
- Conner, M. W., Flood, W. H., Rogers, A. E., and Amdur, M. O. (1988). Lung injury in guinea pigs caused by multiple exposures to ultrafine zinc oxide: Changes in pulmonary lavage fluid. *J. Toxicol. Environ. Health* **25**, 57–69.
- Cosma, G., Fulton, H., DeFeo, T., and Gordon, T. (1992). Rat lung metallothionein and heme oxygenase gene expression following ozone and zinc oxide exposure. *Toxicol. Appl. Pharmacol.* **117**, 75–80.
- De, S. K., McMaster, M. T., and Andrews, G. K. (1990). Endotoxin induction of murine metallothionein gene expression. *J. Biol. Chem.* **265**, 15267–15274.
- Devlin, R. B., Folinsbee, L. J., Biscardi, F., Hatch, G., Becker, S., Madden, M. C., Robbins, M., and Koren, H. L. (1997). Inflammation and cell damage induced by repeated exposure of humans to ozone. *Inhal. Toxicol.* **9**, 211–235.
- Donham, K. J. (1986). Hazardous agents in agricultural dusts and methods of evaluation. *Am. J. Ind. Med.* **10**, 205–220.
- Drinker, P., Thomson, R. M., and Finn, J. L. (1927). Metal fume fever: II. Resistance acquired by inhalation of zinc oxide on two successive days. *J. Ind. Hyg.* **9**, 98–105.
- Dryson, E. W., and Rogers, D. A. (1991). Exposure to fumes in typical New Zealand welding operations. *N. Z. Med. J.* **104**, 365–367.
- Elder, A. C., Finkelstein, J., Johnston, C., Gelein, R., and Oberdörster, G. (2000). Induction of adaptation to inhaled lipopolysaccharide in young and old rats and mice. *Inhal. Toxicol.* **12**, 225–243.
- Fine, J. M., Gordon, T., Chen, L. C., Kinney, P., Falcone, G., and Beckett, W. S. (1997). Metal fume fever: Characterization of clinical and plasma IL-6 responses in controlled human exposures to zinc oxide fume at and below the threshold limit value. *J. Occup. Environ. Med.* **39**, 722–726.
- Flaherty, D. K., Deck, F. H., Cooper, J., Bishop, K., Winzenburger, P. A., Smith, L. R., Bynum, L., and Witmer, W. B. (1984). Bacterial endotoxin isolated from a water spray air humidification system as a putative agent of occupation-related lung disease. *Infect. Immun.* **43**, 206–212.
- Flohé, S., Dominguez Fernandez, E., Ackermann, M., Hirsch, T., Börgermann, J., and Schade, F. U. (1999). Endotoxin tolerance in rats: Expression of TNF- α , IL-6, IL-10, VCAM-1, and HSP 70 in lung and liver during endotoxin shock. *Cytokine* **11**, 796–804.
- Folz, R. J., Abushama, A. M., and Suliman, H. B. (1999). Extracellular superoxide dismutase in the airways of transgenic mice reduces inflammation and attenuates lung toxicity following hyperoxia. *J. Clin. Invest.* **103**, 1055–1066.
- Gordon, T. (1992). Dose-dependent pulmonary effects of inhaled endotoxin in guinea pigs. *Environ. Res.* **59**, 416–426.
- Gordon, T., Balmes, J., Fine, J., and Sheppard, D. (1991). Airway oedema and obstruction in guinea pigs exposed to inhaled endotoxin. *Br. J. Ind. Med.* **48**, 629–635.
- Gordon, T., Chen, L. C., Fine, J. M., Schlesinger, R. B., Su, W. Y., Kimmel, T. A., and Amdur, M. O. (1992). Pulmonary effects of inhaled zinc oxide in human subjects, guinea pigs, rats, and rabbits. *Am. Ind. Hyg. Assoc. J.* **53**, 503–509.
- Hudak, B. B., Zhang, L.-Y., and Kleeberger, S. R. (1993). Inter-strain variation in susceptibility to hyperoxic injury of murine airways. *Pharmacogenetics* **3**, 135–143.
- Hudson, A. R., Kilburn, K. H., Halprin, G. M., and McKenzie, W. N. (1977). Granulocyte recruitment to airways exposed to endotoxin aerosols. *Am. Rev. Respir. Dis.* **115**, 89–95.
- Hur, T., Squibb, K., Cosma, G., Horowitz, S., Piedboeuf, B., Bowser, D., and Gordon, T. (1999). Induction of metallothionein and heme oxygenase in rats after inhalation of endotoxin. *J. Toxicol. Environ. Health* **56**, 183–203.
- Jörres, R. A., Holz, O., Zachgo, W., Timm, P., Koschyk, S., Müller, B., Grimminger, F., Seeger, W., Kelly, F. J., Dunster, C., Frischer, T., Lubeck, G., Waschewski, M., Niendorf, A., and Magnussen, H. (2000). The effect of repeated ozone exposures on inflammatory markers in bronchoalveolar lavage fluid and mucosal biopsies. *Am. J. Respir. Crit. Care Med.* **161**, 1855–1861.
- Karin, M., Imbra, R. J., Heguy, A., and Wong, G. (1985). Interleukin-1 regulates human metallothionein gene expression. *Mol. Cell. Biol.* **5**, 2866–2869.
- Kikuchi, Y., Irie, M., Kasahara, T., Sawada, J., and Terao, T. (1993). Induction of metallothionein in a human astrocytoma cell line by interleukin-1 and heavy metals. *FEBS Lett.* **317**, 22–26.
- Klaassen, C. D., and Lehman-McKeeman, L. D. (1989). Induction of metallothionein. *J. Am. Coll. Toxicol.* **8**, 1316–1321.
- Klaassen, C. D., and Liu, J. (1998). Induction of metallothionein as an adaptive mechanism affecting the magnitude and progression of toxicological injury. *Environ. Health Perspect.* **106**(Suppl. 1), 297–300.
- Kleeberger, S. R., and Hudak, B. B. (1992). Acute ozone-induced change in airway permeability: Role of infiltrating leukocytes. *J. Appl. Physiol.* **72**, 670–676.
- Kuschner, W. G., D'Alessandro, A., Wong, H., and Blanc, P. D. (1997). Early pulmonary cytokine responses to zinc oxide fume inhalation. *Environ. Res.* **75**, 7–11.
- Laughlin, M. J., Wild, L., Nickerson, P. A., and Matalon, S. (1986). Effects of hyperoxia on alveolar permeability of neutropenic rabbits. *J. Appl. Physiol.* **61**, 1126–1131.
- Li, X. Y., Donaldson, K., Brown, D., and MacNee, W. (1995). The role of

- tumor necrosis factor in increased airspace epithelial permeability in acute lung inflammation. *Am. J. Respir. Cell. Mol. Biol.* **13**, 185–195.
- Liu, J., Liu, Y. P., Sendelbach, L. E., and Klaassen, C. D. (1991). Endotoxin induction of hepatic metallothionein is mediated through cytokines. *Toxicol. Appl. Pharmacol.* **109**, 235–240.
- Martin, C. J., Le, X. C., Guidotti, T. L., Yalcin, S., Chum, E., Audette, R. J., Liang, C., Yuan, B., Zhang, X., and Wu, J. (1999). Zinc exposure in Chinese foundry workers. *Am. J. Ind. Med.* **35**, 574–580.
- McCarthy, J. F., Yurek, G. J., Elliott, J. F., and Amdur, M. O. (1982). Generation and characterization of submicron aerosols of zinc oxide. *Am. Ind. Hyg. Assoc. J.* **43**, 880–886.
- McKinney, W. J., Jaskot, R. H., Richards, J. H., Costa, D. L., and Dreher, K. L. (1998). Cytokine mediation of ozone-induced pulmonary adaptation. *Am. J. Respir. Cell. Mol. Biol.* **18**, 696–705.
- Min, K.-S., Terano, Y., Onosaka, S., and Tanaka, K. (1991). Induction of hepatic metallothionein by nonmetallic compounds associated with acute-phase response in inflammation. *Toxicol. Appl. Pharmacol.* **111**, 152–162.
- O'Leary, E. C., Marder, P., and Zuckerman, S. H. (1996). Glucocorticoid effects in an endotoxin-induced rat pulmonary inflammation model: Differential effects on neutrophil influx, integrin expression, and inflammatory mediators. *Am. J. Respir. Cell. Mol. Biol.* **15**, 97–106.
- Palmiter, R. D. (1998). The elusive function of metallothioneins. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8428–8430.
- Piedboeuf, B., Johnston, C. J., Watkins, R. H., Hudak, B. B., Lazo, J. S., Cherian, M. G., and Horowitz, S. (1994). Increased expression of tissue inhibitor of metalloproteinases (TIMP-I) and metallothionein in murine lungs after hyperoxic exposure. *Am. J. Respir. Cell. Mol. Biol.* **10**, 123–132.
- Raj, J. U., Hazinski, T. A., and Bland, R. D. (1985). Oxygen-induced lung microvascular injury in neutropenic rabbits and lambs. *J. Appl. Physiol.* **58**, 921–927.
- Reinhart, P. G., Bassett, D. J., and Bhalla, D. K. (1998). The influence of polymorphonuclear leukocytes on altered pulmonary epithelial permeability during ozone exposure. *Toxicology* **127**, 17–28.
- Rose, C. S., and Blanc, P. D. (1998). Inhalation fevers. In *Environmental and Occupational Medicine* (W. N. Rom, Ed.), pp. 373–380. Lippincott-Raven Publishers, Philadelphia.
- Rylander, R. (1990). Health effects of cotton dust exposures. *Am. J. Ind. Med.* **17**, 39–45.
- Sadhu, C., and Gedamu, L. (1988). Regulation of human metallothionein (MT) genes. Differential expression of MTI-F, MTI-G, and MTII-A genes in the hepatoblastoma cell line (HepG2). *J. Biol. Chem.* **263**, 2679–2684.
- Saltini, C., Hance, A. J., Ferrans, V. J., Bassett, F., Bitterman, P. B., and Crystal, R. G. (1984). Accurate quantification of cells recovered by bronchoalveolar lavage. *Am. Rev. Respir. Dis.* **130**, 650–658.
- Sato, M., and Bremner, I. (1993). Oxygen-free radicals and metallothionein. *Free Radic. Biol. Med.* **14**, 325–337.
- Sato, M., Sasaki, M., and Hojo, H. (1992). Tissue specific induction of metallothionein synthesis by tumor necrosis factor- α . *Res. Commun. Chem. Pathol. Pharmacol.* **75**, 159–172.
- Schroeder, J. J., and Cousins, R. J. (1990). Interleukin-6 regulates metallothionein gene expression and zinc metabolism in hepatocyte monolayer cultures. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3137–3141.
- Schwartz, D. A., Thorne, P. S., Jagielo, P. J., White, G. E., Bleuer, S. A., and Frees, K. L. (1994). Endotoxin responsiveness and grain dust-induced inflammation in the lower respiratory tract. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **267**, 609–617.
- Shimada, M., Tsukada, H., Ishizuka, O., Kon, Y., Hasegawa, T., Suzuki, E., and Gejyo, F. (2000). Lipopolysaccharide tolerance in relation to intrabronchial influx of neutrophils in the rat. *Lung* **178**, 235–248.
- Sturgis, C. C., Drinker, P., and Thomson, R. M. (1927). Metal fume fever: I. Clinical observations on the effect of the experimental inhalation of zinc oxide by two apparently normal persons. *J. Ind. Hyg.* **9**, 88–97.
- Tepper, J. S., Costa, D. L., Lehmann, J. R., Weber, M. F., and Hatch, G. E. (1989). Unattenuated structural and biochemical alterations in the rat lung during functional adaptation to ozone. *Am. Rev. Respir. Dis.* **140**, 493–501.
- Thornalley, P. J., and Vasak, M. (1985). Possible role for metallothionein in protection against radiation-induced oxidative stress: Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochim. Biophys. Acta* **827**, 36–44.
- Turner, J. A., and Thompson, L. R. (1926). Health hazards of brass foundries. *Public Health Bull.* **157**, 1–71.
- Waalkes, M. P., and Goering, P. L. (1990). Metallothionein and other cadmium-binding proteins: Recent developments. *Chem. Res. Toxicol.* **3**, 281–288.
- Wahl, G. M., Stern, M., and Stark, G. R. (1979). Efficient transfer of large DNA fragments from agarose gels to dioxybenzoyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3683–3687.
- Wiener-Kronish, J. P., Albertine, K. H., and Matthay, M. A. (1991). Differential responses of the endothelial and epithelial barriers of the lung in sheep to *Escherichia coli* endotoxin. *J. Clin. Invest.* **88**, 864–875.
- Wiester, M. J., Winsett, D. W., Richards, J. H., Jackson, M. C., Crissman, K. M., and Costa, D. L. (2000). Ozone adaptation in mice and its association with ascorbic acid in the lung. *Inhal. Toxicol.* **12**, 577–590.