Increased Pulmonary Response to Inhaled Endotoxin in Lactating Rats

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An important aspect of risk assessment is identification of subpopulations particularly susceptible to the effects of inhaled pollutants. The present study examined whether female rats were more sensitive during lactation to the acute pulmonary injury produced by inhaled endotoxin. Lactating and age-matched virgin female rats were exposed to aerosols of saline or endotoxin for 3 h and lavaged at 24 h after exposure. No significant differences in lactate dehydrogenase, β-glucuronidase, total protein, and total cell and PMN counts were observed between virgin and lactating rats after exposure to saline. Each marker of pulmonary injury except β-glucuronidase was 1.5- to 3-fold greater in lactating than in virgin female rats exposed to 29.6 $\mu g/m^3$ endotoxin. PMNs (6-fold), total cell counts, and protein were also significantly increased ($p < 0.05$) in lactating rats exposed to 1.3 $\mu g/m^3$ endotoxin, a concentration reported to occur in a number of agricultural settings. These results demonstrate that the physiologic state of lactation is associated with an increased sensitivity to the acute pulmonary injury produced by inhaled endotoxin and are consistent with previous work demonstrating a similar increased sensitivity to ozone exposure. The possibility of a similar pattern of enhanced response in analogous groups of humans merits examination.

Identification of subpopulations that are hypersensitive to the adverse effects of inhaled pollutants is a research category needful of a greater effort. Few studies have attempted to identify those individuals who are at a greater risk or the mechanisms underlying the acquired or genetic basis for the heightened risk. Identification of sensitive subpopulations and understanding the physiologic, cellular, or molecular basis for the heightened response can be critical in prevention and treatment of the adverse effects of airborne pollutants.

Previous work in this laboratory has demonstrated an age-dependence in the acute pulmonary response to ozone. The lavage fluid recovered from the lungs of neonate rats exposed to 1 ppm ozone had significantly greater amounts of necrotic cells and prostanoids than did that of similarly exposed adults (1). Recently, we have extended these investigations to examine the effect of the physiologic states of pregnancy and lactation on the pulmonary response to ozone. These latter studies have demonstrated that significantly greater increases in polymorphonuclear leukocytes (PMNs) and protein are recovered in the lavage fluid of rats in the late stage of pregnancy and throughout lactation in comparison with age-matched virgin female rats after a single 6-h exposure to 1.0 ppm ozone (2).

The mechanisms responsible for the increased sensitivity of pregnant or lactating animals to inhaled pollutants are not clear. There is a small body of published data which suggests that pregnancy induces a metabolic state that imparts sensitivity to oxidants. Lachant and coworkers (3) reported that erythrocytes of pregnant women acquire an increased susceptibility to oxidant damage that is possibly due to a reduced glutathione content and an impaired pentose phosphate shunt. A second line of evidence consistent with susceptibility to oxidative changes during pregnancy is the higher level of circulating serum lipid peroxides reported in pregnant women and rats compared with that in nonpregnant control subjects (4, 5). These metabolic changes or responses of pregnant animals and women appear to be reflective of an endogenous state that is more sensitive to the effects of oxidants than that of adult females experiencing normal ovulatory cycling.

Endotoxin is a ubiquitous agent that manifests some of its toxicity through oxidative reactions involving the induction of a respiratory burst by phagocytic cells. Several animal studies have demonstrated an enhanced sensitization to endotoxin during the physiologic state of pregnancy, as reviewed in reference 6 by Stark and Jackson. These investigators suggested that "prooxidant" metabolic changes during pregnancy might explain this phenomenon of sensitization. Although some of these metabolic changes subside after parturition, it is likely that others continue throughout the physiologic state of lactation.

In the study reported here, we sought to determine whether during lactation the mammalian pulmonary system demonstrates an increased sensitivity to inhaled endotoxin, a common occupational airborne pollutant. Pregnancy and lactation are normal physiologic states for a large portion of the population, and they constitute a sizeable subpopulation that may undergo exposure to elevated concentrations of endotoxin-contaminated organic dusts in textile mills (7), agricultural operations such as grain handling and mulching (8, 9), in swine and poultry confinement buildings, and in automotive milling operations (10). The present study, therefore, compared the sensitivity of lactating and age-matched virgin female rats to the pulmonary injury and inflammation produced by aerosolized endotoxin.

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METHODS

Experimental Animals

Viral antibody-free Sprague Dawley female rats were purchased from Charles River Laboratories (Kingston, NY). Rats were given standard laboratory rodent chow (Purina, St. Louis, MO) and tap water ad libitum and housed with a 12-h light/dark cycle. Lactation time was calculated as the number of days after parturition.

Experimental Design

Lactating rats (first time litters, Days 16 to 19 of lactation) were exposed to aerosolized saline or endotoxin for 3 h (n = 5, 6, and 8 for the saline, low-, and high-concentration endotoxin lactating animals, respectively). Litters were removed during exposure. Age-matched virgin female rats were exposed in an identical manner (n = 7, 6, and 8 for the saline, low-, and high-concentration endotoxin virgin female rats, respectively). Lungs were lavaged at 24 h after the initiation of exposure, and biochemical and cellular components in lavage were measured for the purpose of assessing pulmonary injury.

Inhalation Exposures

Individual animals were separated by wire mesh caging and exposed to aerosolized saline or endotoxin in acrylic exposure chambers. Aerosols were generated with a Babington-type nebulizer (Solosphere; Airlife Inc., Modesto, CA) driven by medical-grade breathing air at 9 psi. The output of the nebulizer was diluted with 10 L/min charcoal and HEPA-filtered air prior to entering the exposure chamber. Airborne endotoxin concentrations in the exposure chamber were varied by adding 10-fold serial dilutions of endotoxin to the nebulizer. Solutions of endotoxin (*E. coli* 0127:B8; Difco Laboratories, Detroit, MI) were made up immediately prior to exposure with pyrogen-free, sterile saline (Baxter Laboratories, Deerfield, IL). The nebulizer reservoir was emptied and refilled at approximately 60-min intervals during exposure to minimize concentrating effects of the nebulizer. Exposure concentrations were determined by routinely taking samples of the chamber atmosphere in the breathing zone of the animals at approximately 30 and 150 min into the exposure period. Filter samples from aerosolized endotoxin were collected, extracted, and analyzed for endotoxin levels using sterile techniques. All tubes, pipets, pipet tips, and microtiter assay plates were pyrogen-free. Analysis of blank filters and water samples in each assay demonstrated that there was no prior pyrogen contamination. Air samples were taken with glass fiber filters 47 mm in diameter (Type A/E; Gelman Sciences, Ann Arbor, MI) for 5 min at 1.07 L/min. The filter samples were immediately placed in pyrogen-free glass tubes and stored at 4° C until extracted. To extract endotoxin from the filters, 30 ml of sterile, pyrogen-free water (Baxter) were added to each sample. The samples were placed into a water bath at 68° C (11) for 30 min. The extracts were decanted and stored at 4° C until analysis. Samples were extracted and assayed the same day. Endotoxin concentrations were quantitated with a *Limulus* amebocyte lysate assay (LAL) (QC1000; Whittaker Bioproducts, Walkersville, MD) using a spectrophotometric microplate method. The assay results were compared with a standard NBS traceable endotoxin and expressed in terms of endotoxin units (EU) or ng (10 EU were assumed to equal 1 ng). The airborne endotoxin concentrations were expressed in $\mu g/m^3$.

The exposure chamber relative humidity was greater than 75%, and the temperature ranged from 23 to 24° C. The size distribution of the endotoxin particles was determined by taking 10-min samples at 2 L/min with a Marple personal cascade impactor (Andersen Instruments Inc., Atlanta, GA). The mass median aerodynamic diameter was 2.5 μm, with a geometric standard deviation of 1.8.

Bronchoalveolar Lavage

At 24 ± 2 h after the beginning of the exposure period, rats were killed by an overdose of sodium pentobarbital (150 to 250 mg/kg, given intraperitoneally) and exsanguinated via the abdominal aorta. The lungs were lavaged *in situ* six times using calcium- and magnesium-free phosphate-buffered saline at pH 7.2 (Gibco-BRL, Gaithersburg, MD). To prevent injury caused by the lavage procedure itself, the volume of fluid used to lavage the animal's lungs was equal to 70% of TLC. TLC was calculated from body weight by using separate equations generated from data obtained in our laboratory for virgin and lactating female rats. The lavage fluid was centrifuged at 400 × *g*, and aliquots of the supernatant from the first lavage were analyzed the same day for β-glucuronidase and lactate dehydrogenase (LDH) activities with commercially available kits (Sigma Chemical Co., St. Louis, MO), whereas other aliquots were stored at −70° C for total protein analysis by the method of Smith and coworkers (12) using bovine serum albumin as a standard. Supernatants from lavages 2 to 6 were discarded. The pelleted cells from all lavages of an individual animal were pooled and enumerated, and an aliquot of cells from each animal was cytocentrifuged onto a slide, fixed with methanol, and stained with Diff Quick® (Baxter) for cell differential counts. Eosinophils were differentiated from neutrophils by their ropelike, rather than lobulated, nucleus and more prominent cytoplasmic granules. Between 100 and 200 cells were counted for each animal.

Statistical Analysis

Intergroup comparisons were made for each dependent variable (i.e., bronchoalveolar lavage parameter) by a two-way analysis of variance. If a significant interaction between exposure (endotoxin concentration) and physiologic state (lactating versus virgin) was observed, Student's unpaired *t* test using a Bonferroni correction for multiple comparisons was employed to analyze group differences between lactating and virgin animals at each dose of endotoxin. In all cases, statistical significance was accepted at $p \leq 0.05$.

RESULTS

Exposure to aerosols of endotoxin produced changes in biochemical and cellular lavage parameters in the rat lung. Two-way analysis of variance demonstrated that there were statistically significant interactive as well as independent effects of concentration and the physiologic state in this study. Concentration had a significant influence on the outcome of each lavage parameter studied ($0.0001 < p < 0.002$). After the 3-h exposure to the high concentration of endotoxin (29.6 ± 8.0 $\mu g/m^3$, mean ± SD), all lavage parameters in virgin and lactating rats were significantly increased over respective control saline values ($p < 0.05$). After exposure to the low concentration of endotoxin (1.3 ± 0.1 $\mu g/m^3$), significant increases in protein, total cells, and PMNs over respective

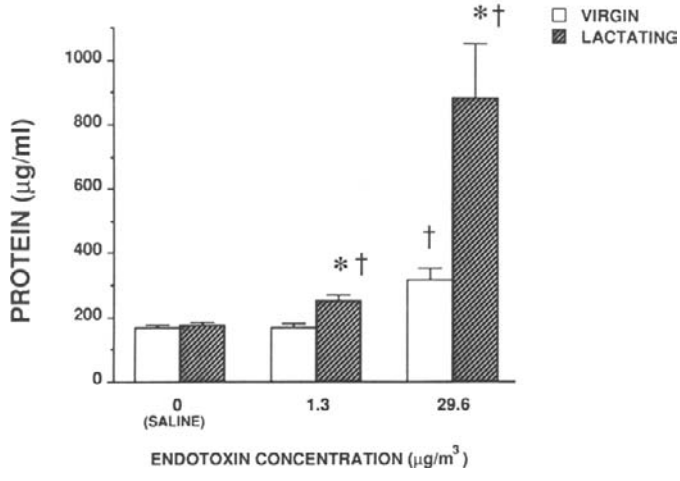

Figure 1. Effect of a 3-h inhalation of aerosolized saline or endotoxin (1.3 or 29.6 $\mu g/m^3$) on the mean (± SE) protein content (μg/ml) in lavage fluid of lactating (*hatched columns*) and age-matched virgin (*open columns*) female rats at 24 h after exposure. * Statistically different from the age-matched virgin rats in the same exposure category. † Statistically different from the saline-exposed animals of the same physiologic state (virgin or lactating).

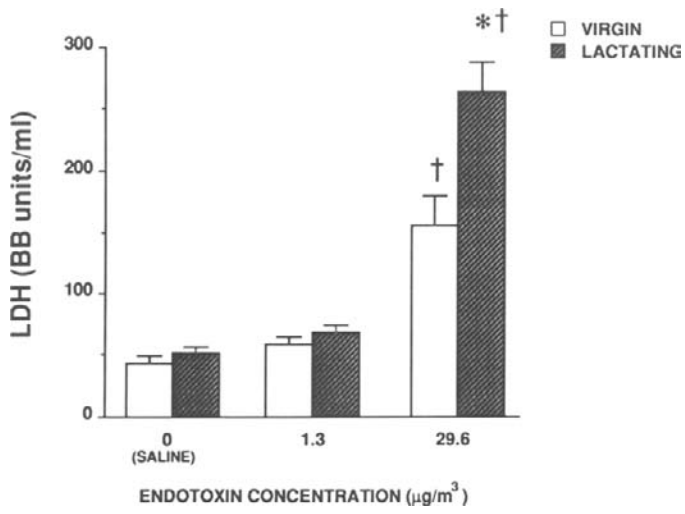

Figure 2. Effect of a 3-h inhalation of aerosolized saline or endotoxin (1.3 or 29.6 μg/m³) on the mean (± SE) LDH activity (BB units/ml) in lavage fluid of lactating (*hatched columns*) and age-matched virgin (*open columns*) female rats at 24 h after exposure. * Statistically different from the age-matched virgin rats in the same exposure category. † Statistically different from the saline-exposed animals of the same physiologic state (virgin or lactating).

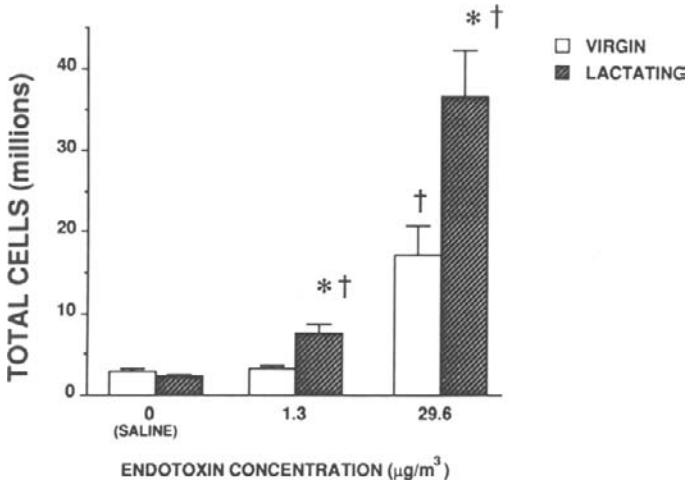

Figure 3. Effect of a 3-h inhalation of aerosolized saline or endotoxin (1.3 or 29.6 μg/m³) on the mean (± SE) total cell count (millions) in lavage fluid of lactating (*hatched columns*) and age-matched virgin (*open columns*) female rats at 24 h after exposure. * Statistically different from the age-matched virgin rats in the same exposure category. † Statistically different from the saline-exposed animals of the same physiologic state (virgin or lactating).

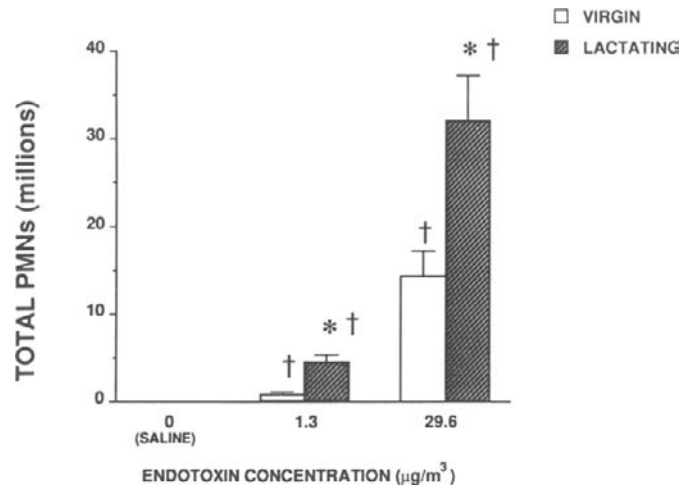

Figure 4. Effect of a 3-h inhalation of aerosolized saline or endotoxin (1.3 or 29.6 μg/m³) on the mean (± SE) PMNs (millions) in lavage fluid of lactating (*hatched columns*) and age-matched virgin (*open columns*) female rats at 24 h after exposure. * Statistically different from the age-matched virgin rats in the same exposure category. † Statistically different from the saline-exposed animals of the same physiologic state (virgin or lactating). Because of the scale of the Y-axis, the mean (± SE) values for the saline-exposed virgin (0.0 ± 0.0) and lactating (0.03 ± 0.01) animals do not appear.

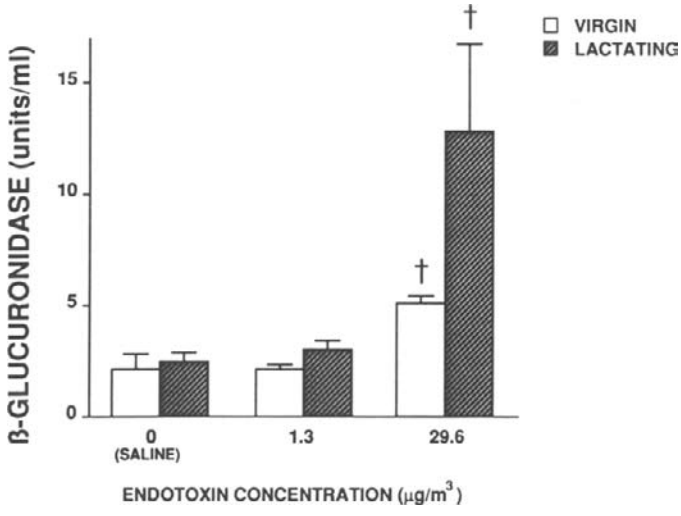

Figure 5. Effect of a 3-h inhalation of aerosolized saline or endotoxin (1.3 or 29.6 μg/m³) on the mean (± SE) β-glucuronidase (units/ml) in lavage fluid of lactating (*hatched columns*) and age-matched virgin (*open columns*) female rats at 24 h after exposure. † Statistically different from the saline-exposed animals of the same physiologic state (virgin or lactating).

control values were obtained only for lactating animals (see figures 1–5).

Analysis of variance demonstrated that the physiologic state (lactation or virgin) also had a significant influence on the effect of inhaled endotoxin ($0.004 < p < 0.01$ for all parameters except β-glucuronidase where $p = 0.08$). Although no significant differences in any of the examined lavage parameters were observed between lactating and virgin rats exposed to a saline aerosol, the observed pulmonary injury after exposure to the high concentration of endotoxin was enhanced in lactating female rats when compared with that in age-matched virgin female rats for all parameters except β-glucuronidase ($p < 0.05$) (figures 1–5). The influence of lactation on the pulmonary response to inhaled endotoxin appeared to be greatest for the cellular influx. The majority of the increase in total cells recovered in the lavage fluid of lactating and virgin animals was due to a tremendous influx of PMNs into the air spaces of the lung. The percentage of PMNs in the lavage cell pellet ranged from zero to 3% in the saline-exposed animals (eight of 12 animals had no observed PMNs) and from 78 to 94% in the high-concentration endotoxin-exposed animals. The absolute number of PMNs migrating into the air spaces was significantly greater in lactating than in virgin female animals, as shown in fig-

ure 4. The inflammatory response of the low-concentration endotoxin-exposed lactating animals was also greater than that of the endotoxin-exposed virgin animals. Significantly greater changes in total protein (51% increase), lavageable cells (134% increase), and PMNs (513% increase) occurred in lactating rats than in age-matched virgin rats in response to the low-concentration endotoxin exposure ($p < 0.05$) (figures 1, 3, and 4). Regardless of exposure, other than PMNs, only mononuclear cells were observed in the recovered lavage cells. No significant differences were observed for cell viability or lavage fluid recovery. The mean viability of cells ranged from 91 to 98%. The recovery of lavage fluid from the first lavage was 80 ± 4% for virgin animals and 80 ± 2% for lactating animals.

DISCUSSION

Occupational exposure to airborne organic dusts contaminated with endotoxin is associated with a variety of adverse pulmonary effects (13). Although many of these inflammatory effects of inhaled endotoxin have been examined in animal models, the present study is the first to demonstrate that a particular subpopulation may be more sensitive to these injurious effects. An enhanced response was observed in rats during the physiologic state of lactation after a single 3-h exposure to 1.3 μg/m³ endotoxin, a concentration of airborne endotoxin that has been reported to occur in agricultural worksites (8, 9, 14). The most sensitive parameters of the enhanced adverse pulmonary response to inhaled endotoxin in lactating rats were increases in total protein and in PMNs in lavage fluid. Although pathologic and functional end points were not examined in this study, the enhanced response appeared to occur as a result of injury in the alveolar region. Plasma proteins are the likely source of the increase in total protein in lavage fluid (15), whereas a number of inflammatory mediators released by cells in the respiratory tract in response to endotoxin are chemoattractants for PMNs. The mean percentage of PMNs in the cells recovered in the lavage fluid increased from less than 1.6% in saline-exposed lactating animals to 58 and 88% in the low- and high-concentration endotoxin-exposed lactating animals, respectively. More importantly, the absolute number of PMNs recovered in lavage fluid was 6-fold greater in the lactating animals than in the age-matched virgin animals after exposure to the low concentration of endotoxin. LDH activity in the lavage fluid was also significantly greater in the high-concentration endotoxin-exposed lactating rats, suggesting that respiratory cell injury occurred along with the migration of protein and PMNs from the vasculature. These results are similar to previous work performed in this laboratory (2) which demonstrated that lactating and pregnant rats are more sensitive than virgin rats of the same age to the adverse pulmonary effects of ozone.

The physiologic state of lactation appears to sensitize the lung to two diverse inhaled agents; however, a common mechanism underlying the enhanced response is not clear. Several physiologic and biochemical factors may play a role in this enhanced response to inhaled endotoxin in lactating rats. As in pregnancy, the metabolic load is increased significantly during lactation, and it may alter the pattern or frequency of breathing. A change in the depth of breathing or an increase in minute ventilation, such as seen in women during pregnancy (16), would favor increased particle deposition and thus increase the retained dose of endotoxin in the lung. In women, however, the increase in minute ventilation observed during pregnancy appears to rapidly return to normal values postpartum (16, 17). Unfortunately, these reports do not differentiate the postpartum state into lactation and nonlactation, and thus it is not known whether a return of minute ventilation to prepregnancy values occurs in both lactating and nonlactating mothers. We are not aware of any reports on ventilation in rats during pregnancy and lactation. One study, however, did examine pulmonary mechanics in rats during pregnancy and lactation, and it demonstrated that similar to the rapid recovery of minute ventilation in women after parturition, the retractive forces of the lung that were increased during pregnancy in rats recovered within 2 to 3 wk after delivery (18). Because no other pulmonary function measurements were made in that study, however, it is not clear whether a change in tidal volume and minute ventilation contributed to the observed enhanced pulmonary injury in lactating rats in the present study.

Several other metabolic and hormonal changes occur during lactation that might account for the enhanced pulmonary injury observed after inhalation of aerosolized endotoxin. Antioxidants have been proposed to play a role in the protection of the lung against oxidant injury, and therefore deficiencies in antioxidant vitamins during lactation might account for the increased susceptibility to inhaled agents that cause injury by oxidative pathways. Although vitamin E status was acceptable, deficiencies in essential nutrients such as ascorbic acid, beta-carotene, and vitamin A were reported for healthy women for as long as 10 wk postpartum (19). Similarly, a decline in plasma ascorbic acid has been reported to occur during both pregnancy and lactation (20).

Another consideration for the cause of the increased sensitivity to endotoxin during lactation is an increase in metabolic rate, which may affect the metabolic changes induced by endotoxin. Oxygen consumption is known to increase progressively during pregnancy, reaching values approximately 30 to 40% above pregestational levels (21). The increased metabolic rate likely continues during the increased physiologic demands of lactation. Evidence in mice demonstrates that total oxygen consumption is elevated by approximately 50% during lactation (22). Although oxygen consumption/carbon dioxide production has been reported to decrease in women after parturition (16, 17), the investigators did not state whether the subjects were lactating or not. Thus, data on oxygen consumption specifically for lactating women do not appear to exist.

Many factors must be considered in examining the relevance of the enhanced pulmonary injury observed in the present study to actual human exposures and risk assessment. Direct extrapolation from dose-response data in lactating rats to relevant exposure concentrations across species is difficult. For example, the results of the present study and previous work from this laboratory demonstrate that the rat is relatively more resistant to the injurious effects of inhaled endotoxin than is the guinea pig (23). Whereas no significant changes in LDH activity was observed in virgin rats at 1.3 μg/m³ endotoxin, significant increases were observed in guinea pigs at concentrations as low as 0.05 μg/m³. On the other hand, preliminary results from this laboratory have demonstrated that the state of lactation is associated with increased ozone-induced pulmonary injury in more than one rodent species: lactating mice are more sensitive to the injurious effects of 1.0 ppm ozone than are virgin female mice (unpublished data). Thus, the results from the present and previous studies in this laboratory strongly suggest that the physiologic states of lactation and pregnancy are associated with enhanced susceptibility of the mammalian lung to pollutant-induced injury. Another factor to be considered in extrapolation of our results is a difference in particle type and size between aerosols encountered in the real world and those used in the present study. Occupationally encountered dusts have many components. The storage and handling conditions that promote the growth of gram-negative bacteria also support the growth of many other microbial agents capable

of eliciting a pulmonary response. In addition, endotoxin-contaminated dusts in the workplace have a large size distribution and therefore may have a different pattern of deposition than the fairly narrow distribution of particles used in this study (geometric standard deviation of 1.8).

As of 1988, the average number of live births per woman in the United States was 1.9 (24). Thus, a woman may spend several months in the latter stages of pregnancy and lactation during which she may have heightened sensitivity to inhaled pollutants in general. Although women can encounter airborne endotoxin in the home (swamp cooler-type air conditioners and nebulizing humidifiers), the highest concentrations of endotoxin exposure occur in occupational settings. Several investigators have measured airborne endotoxin concentrations above 1 $\mu g/m^3$ in agricultural settings (8, 9, 14). Thus, although the concentrations of airborne endotoxin used in this study (1 to 30 $\mu g/m^3$) were at the upper end of values reported for agricultural settings, the possibility of inadvertent exposure to these levels of endotoxin is great. Moreover, although enhanced pulmonary injury may occur in lactating women from single exposures to these upper-end exposure levels of airborne endotoxin, a more likely exposure regimen and important consideration for risk assessment is the effect of repeated exposure to lower levels of endotoxin-contaminated organic aerosols in the workplace.

In conclusion, significantly greater pulmonary injury was produced by inhaled endotoxin in the pulmonary system of lactating rats than in virgin female rats. This enhancement of injury suggests that lactating women may be at greater risk to the adverse effects of endotoxin-contaminated particles encountered in the workplace and at home. The findings reported here for endotoxin are similar to the results of previous studies demonstrating that lactating as well as pregnant rats are hypersensitive to the adverse effects of ozone (2). Thus, the physiologic state of lactation is associated with increased pulmonary injury from exposure to at least two different inhaled toxicants. Because of the large numbers of lactating and/or pregnant women who may be exposed to environmental and occupational pollutants via the inhalation route, understanding the underlying causes of this enhanced response is critical.

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