Genetic variability in the development of pulmonary tolerance to inhaled pollutants in inbred mice

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Wesselkamper, Scott C., Lung Chi Chen, Steven R. Kleeberger, and Terry Gordon. Genetic variability in the development of pulmonary tolerance to inhaled pollutants in inbred mice. Am J Physiol Lung Cell Mol Physiol 281: L1200–L1209, 2001.—After repeated exposures, many individuals develop tolerance to the adverse health effects of inhaled pollutants. Pulmonary tolerance can be characterized as the ability of the lung to withstand the adverse actions of a toxic compound after repeated exposures. To determine whether genetic background is important to the development of pulmonary tolerance to inhaled pollutants, 11 inbred strains of mice were exposed once (1×) or for 5 consecutive days (5×) to 1.0 mg/m³ of zinc oxide (ZnO). Development of pulmonary tolerance was assessed by measuring polymorphonuclear leukocyte and protein levels in bronchoalveolar lavage fluid and comparing the responses of the 1× and 5× groups. Significant interstrain variation in polymorphonuclear leukocyte and protein responses was observed between the groups with 1× and 5× exposures, which indicates that genetic background has an important role in the development of pulmonary tolerance. The BALB/cByJ strain and the DBA/2J strain were the most tolerant and nontolerant, respectively. The CByD2Fl/J offspring were uniformly nontolerant. The development of tolerance was also investigated in BALB/cByJ and DBA/2J mice after 1× and 5× exposure to ozone and aerosolized endotoxin. Discordance in the phenotypic pattern of pulmonary tolerance among strains after exposure to ZnO, ozone, and endotoxin suggested that different mechanisms may be responsible for the development of pulmonary tolerance to these agents.

inhalation exposure. Tolerance can be defined as the ability to resist the injurious actions of a toxicant after continuous or repeated exposures. Tolerance has many implications for human health, with potentially advantageous and disadvantageous consequences. For example, individuals can be exposed to zinc oxide (ZnO) fumes from welding processes (11), and acquisition of tolerance to the symptomatic effects of ZnO inhalation has been reported in clinical studies of metal fume fever (10, 14) and in a study of zinc foundry workers (42). Pulmonary tolerance to the acute respiratory effects of ozone (O₃) has also been demonstrated in human subjects (8, 12, 17, 23, 24, 27). Similar to the effect of repeated ZnO inhalation, induction of tolerance after repeated systemic endotoxin exposure is a favorable consequence that may avert the occurrence of septic shock (59). On the other hand, acquisition of symptomatic tolerance to repeated exposure to endotoxins associated with cotton dust (9) and contaminated humidifiers (15) may occur initially but later may manifest as chronic lung disease (31, 60).

In general, little is actually known about the molecular mechanism(s) of tolerance. Because the acquisition of pulmonary tolerance from repeated exposure to inhaled toxicants has ramifications for human health, the identification of innate and extrinsic factors that influence the ability to develop tolerance is a significant issue. Interindividual variability exists in the sensitivity to adverse respiratory effects from inhaled toxicants (1, 7, 40, 44), suggesting that genetic background is a host factor for susceptibility. With regard to the phenotype of pulmonary tolerance, a study from our laboratory (14) has demonstrated that individuals vary in their initial response to inhaled ZnO as well as in their ability to develop systemic (e.g., symptoms and fever) and pulmonary tolerance. Similarly, interindividual variability has been shown in the development and persistence of pulmonary tolerance to O₃ (12, 27). These studies suggest gene-environment interactions in human subjects.

Inhalation of ZnO has been shown to cause a marked inflammatory influx of polymorphonuclear leukocytes (PMNs) as well as changes in epithelial permeability in the lungs of humans and animals (4, 13, 22, 38, 39). Exposure to ZnO fumes is also a major occupational respiratory hazard in many industrial processes (41).
In the present study, we hypothesize that genetic background contributes to the acquisition of pulmonary tolerance to inhaled pollutants. Because inbred strains of mice are virtually identical at all loci and share several chromosomal regions of conserved synteny with humans, they are ideal for investigating genotype-environment interactions. Interstrain differences in the development of pulmonary tolerance were identified by measuring pulmonary inflammation and protein in bronchoalveolar lavage (BAL) fluid in 11 inbred strains of mice exposed singly and repeatedly to ZnO fumes. In addition, the acquisition of pulmonary tolerance was examined in inbred mice exposed singly and repeatedly to O₃ or aerosolized endotoxin. Results from these studies demonstrated a significant genetic component in the ability to develop pulmonary tolerance to inhaled toxicants.

MATERIALS AND METHODS

Strain selection. All inbred mouse strains [A/J, AKR/J, C3H/HeJ, C3H/HeOuJ, BALB/cJ, BALB/cByJ (CB), C57BL/6J, CBA/J, DBA/2J (D2), SJL/J, 129/J, and CByD2F1/J hybrid] were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were acclimated for at least 1 wk before exposure, housed in a positive-pressure environment with a 12:12-h light-dark cycle that started at 6:00 AM, and were provided with water and standard laboratory rodent chow (Purina, Indianapolis, IN) ad libitum except during exposure. The mice were handled in accordance with the standards established by the US Animal Welfare Acts set forth in National Institutes of Health guidelines and by the New York University School of Medicine Division of Laboratory Animal Resources.

Experimental design. To investigate the contribution of genetic background to the development of pulmonary tolerance, mice from 11 inbred strains were exposed to ZnO fumes once (1×) or for 5 successive days (5×) for 3 h/day. All 1× exposures were done on the same day as the final 5× exposure, and control animals were exposed to air. Acquisition of pulmonary tolerance was assessed by measuring inflammatory cellular influx and protein concentration in BAL fluid in a manner similar to that used in other investigations of tolerance to a variety of inhaled agents (2, 6, 20, 53). Previously, our laboratory (56) demonstrated that pulmonary tolerance to PMN influx, but not lavageable protein and lung pathology, is developed in an outbred mouse model after repeated exposure to ZnO. Based on those observations, the pulmonary tolerance phenotype with respect to total BAL fluid PMNs and protein in the present study was characterized as 1) a significant decrease in mean lavageable PMNs between 1× and 5× exposure groups and 2) the absence of cumulative lung injury over the 5-day exposure period as measured by total protein in BAL fluid (no significant increase between the 1× and 5× exposure groups). Two inbred strains with markedly different PMN and protein responses to repeated ZnO exposure were identified: CB (tolerant) and D2 (nontolerant). Total BAL fluid macrophage numbers were also determined in these two strains. To begin assessment of the mode of inheritance of pulmonary tolerance to ZnO exposure, total PMN and protein concentrations from a cross of the tolerant and nontolerant inbred strains (CB × D2F1/J) were measured after 1× and 5× ZnO exposures. Dose-response effects on the development of ZnO-induced pulmonary tolerance were also studied in CB and D2 mice. Lung pathology associated with 1× and 5× exposures to 1.0 mg/m³ of ZnO was determined to illustrate gross histopathological differences between the CB and D2 strains. Furthermore, to evaluate whether the development of the tolerant and nontolerant phenotypes is agent specific, CB and D2 mice were also singly and repeatedly exposed to O₃ and aerosolized endotoxin, and pulmonary tolerance was determined as described above.

Atmosphere generation and characterization. Mice were exposed to ZnO (1.0 ± 0.2 and 2.1 ± 0.2 mg/m³; means ± SD) in stainless steel cages placed inside a 0.07-m³ Plexiglas chamber. ZnO fumes were generated in a furnace as previously described (43, 56). 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-m³ Plexiglas chamber. Endotoxin and saline (control) aerosols were generated with a Babyton-type nebulizer (Solosphere, Airlife, Modesto, CA) driven by high-efficiency particulate air (HEPA)-filtered air at 9 psi. The output of the nebulizer was diluted with HEPA-filtered ambient air before it entered the exposure chamber. Solutions of endotoxin (1.75 and 0.7 μg/ml of lipopolysaccharide, Escherichia coli 0127:B8; Difco Laboratories, Detroit, MI) were made up with pyrogen-free, isotonic saline (0.9% sodium chloride, Baxter Healthcare, Deerfield, IL) immediately before exposure. The nebulizer reservoir was emptied and refilled at 1-h intervals during the exposures to minimize the concentrating effects of the nebulizer.

For O₃ exposures, mice were placed in stainless steel cages inside a 1.0-m³ stainless steel inhalation chamber. O₃ [0.60 ± 0.02 parts/million (ppm)] was generated from 5% oxygen (balance argon) with an ultraviolet ozonator (OREC, Phoenix, AZ). O₃ concentration was monitored in the breathing zone of the chamber with an O₃ detector (Dasibi model 1008-PC, Glendale, CA). This instrument was regularly calibrated against a US Environmental Protection Agency standard. Air (sham) exposures were also conducted for control purposes.

BAL. Twenty-four hours postexposure to ZnO, endotoxin, O₃, air, or saline, mice were killed by intraperitoneal injections of ketamine hydrochloride (100 mg/kg; Vetalar, Fort Dodge Laboratories, Fort Dodge, IA) and pentobarbital sodium (175 mg/kg; Sleepaway, Fort Dodge Laboratories), and the posterior abdominal aorta was severed. The lungs of each mouse were lavaged two times with 1.2 ml of Dulbecco’s phosphate-buffered saline without Ca²⁺ or Mg²⁺ (pH 7.2–7.4, 37°C; GIBCO BRL, Life Technologies, Grand Island, NY). Recovered BAL fluid was immediately placed on ice (4°C). Aliquots (100 μl) of lavage fluid were cytocentrifuged (Cytospin, Shandon Southern Products), and the cells were stained with Hemacolor (EM Science, Gibbstown, NJ), and pentobarbital sodium (175 mg/kg; Sleepaway, Fort Dodge Laboratories), and the posterior abdominal aorta was severed. The lungs of each mouse were lavaged two times with 1.2 ml of Dulbecco’s phosphate-buffered saline without Ca²⁺ or Mg²⁺ (pH 7.2–7.4, 37°C; GIBCO BRL, Life Technologies, Grand Island, NY). Recovered BAL fluid was immediately placed on ice (4°C). Aliquots (100 μl) of lavage fluid were cytocentrifuged (Cytospin, Shandon Southern Products), 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 typical cytological procedures (52). Total cell counts were determined with a hemocytometer. The lavage fluid was then centrifuged (500 g, 7 min, 4°C), and the supernatant was decanted. The total protein concentration in the supernatant was measured with an assay kit (Pierce, Rockford, IL) that employs the method of Bradford (3) and is accurate from 10 to 2,000 μg/ml (bovine serum albumin standard, 550 nm).

Tissue preparation and lung pathology. To obtain tissue for pathological analysis, mice were killed by intraperitoneal injections of pentobarbital sodium (175 mg/kg; Sleepaway), and a cannula (0.6-mm internal diameter) was inserted into the trachea. The lungs were deflated and then infused in situ with 2% glutaraldehyde, removed, and immersed in fixative for at least 24 h. Fixed tissues were dissected, and lung...
sections were dehydrated through a series of ethanol solutions and embedded in paraffin blocks. Paraffin-embedded tissues were sectioned (5 μm), stained with hematoxylin and eosin, and examined by light microscopy. Several lung sections were evaluated for three mice per strain per exposure group, and the reader was blinded to strain and exposure at the time of evaluation. Histopathological changes including inflammatory cell influx, airspace infiltrates, and alveolar epithelium and septal wall thickening were assessed in lung sections from both the CBy and D2 strains.

Data analysis. ZnO-, O₃-, and endotoxin-induced changes in PMNs and protein in BAL fluid are presented as means ± SE. Differences between means were assessed with a two-factor analysis of variance (ANOVA), and the factors for each analysis were strain and exposure. The Student-Newman-Keuls a posteriori test of significance was used to assess differences between means. Significance for all comparisons was accepted at P < 0.05.

RESULTS

Strain phenotype pattern of pulmonary tolerance to repeated ZnO exposure. Total PMN and protein concentrations in BAL returns from 11 inbred mouse strains were used to assess the development of pulmonary tolerance after 1× and 5× exposures to 1.0 ± 0.2 mg/m³ of ZnO (Table 1). Statistical analysis by two-way ANOVA indicated significant effects of strain, exposure, and the interaction of strain and exposure on the mean number of PMNs (x10⁴) and total protein (μg/ml) recovered by BAL (P < 0.05). Based on the aforementioned criteria for pulmonary tolerance, 6 of 11 inbred strains of mice (AKR/J, BALB/cJ, CBy, CBA/J, C3H/HeOuJ, SJL/J, and 129/J) exhibited a tolerant PMN response phenotype after 5× exposure to ZnO. D2 and C3H/HeJ were the only strains that had a nontolerant phenotype with respect to total protein measured in BAL fluid (e.g., D2-1× = 579 ± 55 μg/ml; D2-5× = 1.358 ± 78 μg/ml; means ± SE). Marked interstrain variation in the sensitivity to a 1× ZnO exposure was also observed (Table 1).

Mode of inheritance and dose-response relationship. The CBy and D2 strains were determined to be tolerant and nontolerant, respectively, with regard to total PMN and protein endpoints in BAL fluid after five successive exposures to 1.0 mg/m³ of ZnO. Total PMN and protein concentrations were not significantly different between the CBy and D2 strains after a 1× exposure (P > 0.05). Compared with the 1× exposure group, however, total PMNs returned to baseline values after 5× exposures in the CBy strain but continued to increase in the D2 strain (Fig. 1A). Similarly, ZnO had no effect on macrophage influx or protein in BAL fluid in 1× or 5× exposed CBy mice, but total macrophage and protein concentrations in BAL fluid were significantly increased in 5×-exposed D2 mice compared with those exposed once (Fig. 1, B and C). Total BAL fluid protein concentration and numbers of PMNs and macrophages from D2 mice exposed 5× to ZnO were significantly greater than those of 5× exposed CBy mice. To determine whether the development of pulmonary tolerance is a recessive or dominant trait, the offspring of a cross of the CBy and D2 strains (CByD2F1/J) were exposed 1× and 5× to 1.0 mg/m³ of ZnO. CByD2F1/J mice were nontolerant, with total number of PMNs and protein concentrations not significantly different from those of the D2 progenitor strain after five exposures (P > 0.05; Fig. 1, A and C). These findings suggest that pulmonary tolerance to repeated ZnO exposure is inherited as a recessive trait.

After 1× and 5× exposures to 2.0 mg/m³ of ZnO, the CBy and D2 strains retained the tolerant and nontolerant phenotypes with regard to total PMNs and protein recovered from BAL fluid, respectively. Importantly, compared with a single 1.0 mg/m³ ZnO exposure, CBy mice had a significantly greater total PMNs response after a 1× exposure to 2.0 mg/m³ of ZnO but were tolerant after five exposures (1× = 3.9 ± 1.0 × 10⁴ PMNs; 5× = 0.7 ± 0.2 × 10⁴ PMNs; Fig. 2A). CByD2F1/J mice were nontolerant with respect to total PMN and protein after exposure to 2.0 mg/m³ of ZnO. Unlike the response to 1.0 mg/m³ of ZnO, the total protein in BAL fluid was significantly increased over control values in CBy mice after both the 1× and 5× exposures to 2.0 mg/m³ of ZnO (Fig. 2C) but did not

Table 1. Changes in total PMNs and total protein in BAL fluid of inbred mice 24 h after exposure to ZnO or air for 3 h

<table>
<thead>
<tr>
<th>Strain</th>
<th>1× ZnO</th>
<th>5× ZnO</th>
<th>Air</th>
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<tbody>
<tr>
<td></td>
<td>Total PMNs (x10⁴)</td>
<td>Total protein (μg/ml)</td>
<td>Total PMNs (x10⁴)</td>
</tr>
<tr>
<td>A/J</td>
<td>0.6 ± 0.1 (6)</td>
<td>225 ± 23 (6)</td>
<td>0.4 ± 0.2 (6)</td>
</tr>
<tr>
<td>AKR/J</td>
<td>6.6 ± 3.3 (4)</td>
<td>389 ± 52 (4)</td>
<td>0.7 ± 0.3 (4)</td>
</tr>
<tr>
<td>BALB/cJ</td>
<td>6.0 ± 1.3 (4)</td>
<td>380 ± 32 (4)</td>
<td>0.5 ± 0.2 (4)</td>
</tr>
<tr>
<td>BALB/cByJ</td>
<td>1.1 ± 0.2 (10)</td>
<td>331 ± 18 (10)</td>
<td>0.1 ± 0.01 (24)</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>0.3 ± 0.1 (12)</td>
<td>253 ± 16 (12)</td>
<td>0.6 ± 0.1 (12)</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>0.6 ± 0.2 (6)</td>
<td>141 ± 11 (6)</td>
<td>0.4 ± 0.1 (5)</td>
</tr>
<tr>
<td>C3H/HeOuJ</td>
<td>11.1 ± 2.9 (5)</td>
<td>774 ± 56 (5)</td>
<td>2.2 ± 0.7 (5)</td>
</tr>
<tr>
<td>CBA/J</td>
<td>8.4 ± 1.4 (4)</td>
<td>539 ± 55 (4)</td>
<td>0.9 ± 0.2 (4)</td>
</tr>
<tr>
<td>DBA/2J</td>
<td>1.7 ± 0.3 (14)</td>
<td>579 ± 59 (14)</td>
<td>1.9 ± 0.3 (26)</td>
</tr>
<tr>
<td>SJL/J</td>
<td>10.6 ± 2.0 (6)</td>
<td>1,003 ± 166 (4)</td>
<td>1.8 ± 0.6 (4)</td>
</tr>
<tr>
<td>129/J</td>
<td>1.2 ± 0.2 (4)</td>
<td>282 ± 46 (4)</td>
<td>0.5 ± 0.2 (4)</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses, N. of mice. Twenty-four hours after either single (1×) or repeated (5×; once daily for 5 consecutive days) 3-h exposures to 1.0 mg/m³ of zinc oxide (ZnO) or air (control), changes in total polymorphonuclear leukocytes (PMNs; x10⁴) and total protein (μg/ml) were measured in the bronchoalveolar lavage (BAL) fluid of 11 inbred strains of mice. Significantly different (P < 0.05) from: *air-exposed control mice; †mice in 1× exposure group.
increase over the 5-day exposure period. Additionally, total numbers of macrophages were significantly increased between 1× and 5× exposures to 2.0 mg/m³ of ZnO in the CBy strain (Fig. 2B).

Pulmonary histopathology induced by single and repeated ZnO exposure. Exposure-induced changes in pulmonary histology were evaluated in tolerant CBy mice and nontolerant D2 mice. No marked strain differences in histopathology were observed after a 1× exposure to 1.0 mg/m³ of ZnO (Fig. 3, A and B). Compared with 5× exposed CBy mice, however, more airspace infiltrates (Fig. 3, C and D), alveolar septal wall thickening (Fig. 3, E and F), and alveolar congestion of inflammatory cells were found in lung sections of D2 mice (Fig. 3, C–F). These observations are consistent with the aforementioned ZnO-induced differences in total PMNs, macrophages, and protein in BAL fluid after 1× and 5× exposures.

Pulmonary tolerance to repeated O₃ and aerosolized endotoxin exposure. After 1× and 5× exposures of CBy and D2 mice to 0.6 ppm O₃ or aerosolized endotoxin, total PMN, macrophage, and protein concentrations were used to determine whether the development of pulmonary tolerance was agent specific. Similar to that seen with ZnO exposure, total PMNs were significantly increased over those in control mice after a single exposure to O₃ in the CBy strain, and these mice were tolerant to PMNs and protein in BAL fluid (Fig. 4, A and C). Unlike the response to 5× ZnO exposure, however, total macrophages were significantly increased over control level in 5× O₃-exposed CBy mice (Fig. 4B). Additionally, in the D2 strain, there were very few PMNs in both 1× and 5× O₃-exposed mice (1× = 0.2 ± 0.03 × 10⁴ PMNs; 5× = 0.5 ± 0.2 × 10⁴ PMNs; Fig. 4A) compared with ZnO-exposed mice. Total BAL fluid protein in D2 mice, however, continued to increase between the O₃ exposure groups (1× = 478 ± 39 μg/ml; 5× = 677 ± 25 μg/ml; Fig. 4C) similar to that seen with ZnO exposure, although this increase was not statistically significant (P > 0.05).

With respect to single and repeated exposure to aerosolized endotoxin (generated from a 1.75 μg/ml solution), in the CBy strain, total PMNs were significantly increased over those in control mice after a single exposure, and these mice were tolerant to PMNs in BAL fluid after five successive exposures (Fig. 5A). However, D2 mice, which were nontolerant to ZnO, developed tolerance to repeated endotoxin exposure. The D2 strain exhibited a significant decrease in total PMNs between 1× and 5× endotoxin exposure groups (1× = 4.8 ± 1.1 × 10⁴; 5× = 0.7 ± 0.3 × 10⁴; Fig. 5A). Single and repeated endotoxin exposure had little effect on total BAL fluid macrophage and protein concentrations in CBy and D2 mice compared with those in control mice (Fig. 5, B and C). BAL fluid protein concentrations in the D2 strain, however, significantly
decreased between the 1× and 5× exposure groups (Fig. 5C). Similar results were obtained with a lower concentration of aerosolized endotoxin (generated from a 0.7 mg/ml solution; data not shown).

**DISCUSSION**

In previous human and animal studies, PMNs and protein in BAL fluid have been used as indexes of lung injury to assess the development of pulmonary tolerance after repeated exposure to various inhaled agents. One of the primary objectives of this study was to determine whether the development of pulmonary tolerance to repeated toxicant exposure has a genetic component. Previous investigations (26, 28, 32, 46, 49, 51, 57) have used inbred mouse strains to determine that genetic background plays an important role in susceptibility to oxidant gas- and particle-induced pulmonary inflammation and lung injury. In the present study, we evaluated total PMNs and protein in BAL fluid after 1× or 5× exposures to 2.0 mg/m³ of ZnO or air for 3 h. Values are means ± SE; n = 7–10 mice-strain⁻¹-exposure group⁻¹. Significantly different (P < 0.05) from: *saline-exposed control mice; **1× exposure group; #BALB/cByJ mice.

**Fig. 2.** Total number of PMNs (A) and macrophages (B) and total protein (C) in BAL fluid of BALB/cByJ, DBA/2J, and CByD2F1/J mice 24 h after 1× or 5× exposure to 2.0 mg/m³ of ZnO or air for 3 h. Values are means ± SE; n = 7–10 mice-strain⁻¹-exposure group⁻¹. Significantly different (P < 0.05) from: *saline-exposed control mice; 1× exposure group; #BALB/cByJ mice.
2.0 mg/m³ of ZnO. The CBBy strain retained tolerance to PMNs and protein in BAL fluid after five successive exposures, despite significantly greater PMN and protein responses in 1× exposed mice compared with those of the group exposed to 1.0 mg/m³ of ZnO. Unlike the response elicited by 1.0 mg/m³ of ZnO, however, there was a significant increase in the numbers of macrophages between 1× and 5× exposures to 2.0 mg/m³ of ZnO in CBBy mice. Additionally, the D2 strain remained nontolerant after 1× and 5× exposure to 2.0 mg/m³ of ZnO, but the 1× PMN and protein responses were not different from those of mice exposed to 1.0 mg/m³ of ZnO. These data suggest that in this genetic model, there are dose-response effects of inhaled ZnO on lavageable PMN, protein, and macrophage concentrations in 1× and 5× exposed CBBy and D2 inbred mice. Furthermore, the development of tolerance to cellular influx and protein in BAL fluid after five successive ZnO exposures appears to be independent of the magnitude of the initial 1× responses with the two ZnO exposure concentrations used in this study. It is unknown, however, if tolerance to lavageable PMNs and protein in the CBBy strain is eventually ablated at a higher ZnO exposure concentration.

The mechanism(s) of tolerance to inhaled pollutants is not well understood, and the literature is limited regarding the genes that control this response. Many investigators have hypothesized that the development of pulmonary tolerance to inhaled toxicants may be a result of the production of a multitude of protective...
biological molecules such as antioxidants (58), antioxidant enzymes (18, 30), stress proteins (21, 56), specific cytokines (16, 30, 45), and pulmonary surfactant (47). Additionally, it has been suggested that similar molecular mechanisms may control the development of pulmonary tolerance to repeated toxicant exposure. For example, preexposure to O₃ (29), endotoxin (19), nitrogen dioxide (5), or cadmium aerosol (25) produces cross-tolerance to hyperoxia in rats by reducing lung injury and lethality.

In the present study, CBy and D2 mice were exposed to ZnO, O₃, or aerosolized endotoxin to determine whether the development of pulmonary tolerance is agent specific. Similar to their response to ZnO exposure, the CBy strain was tolerant to increased PMNs and protein in BAL fluid after repeated exposure to O₃ and endotoxin. This suggests that a common mechanism (or collection of genes) may be responsible for the pathogenesis of pulmonary tolerance to repeated toxicant exposure in the CBy strain. Likewise, D2 mice remained nontolerant to protein in BAL fluid after five successive O₃ exposures, although it was unclear whether they were tolerant to O₃-induced PMNs in BAL fluid. In contrast to the response to ZnO exposure, the D2 strain developed tolerance to PMNs and protein in BAL fluid after repeated exposure to aerosolized endotoxin. The disparity in phenotypes between ZnO- and endotoxin-exposed D2 mice is indicative that a different molecular mechanism(s) may regulate the acquisition of pulmonary tolerance to repeated ZnO and endotoxin exposure in the D2 strain. Taken together, these data suggest that the development of tolerance to lung injury from repeated toxicant exposure is a complex trait, and the manifestation of the tolerance phenotype may be agonist dependent.

To identify the candidate genes that may play a role in pulmonary tolerance, we are currently conducting a formal linkage analysis of tolerant and nontolerant phenotypes in segregant populations derived from the CBy and D2 strains. Linkage analyses are expected to produce valuable information on the genetic mechanisms involved in the pathogenesis of pulmonary tolerance, similar to that provided by previous investigations of susceptibility to O₃- and particle-induced lung injury (35, 48, 50, 51). In the present study, the role of the Toll-like receptor 4 (Tlr4) gene in ZnO tolerance was determined by comparing pulmonary responses in C3H/HeJ and C3H/HeOuJ mice as described previously with O₃ and acid-coated particles (36, 37, 49). Similar to what was observed with O₃, the C3H/HeOuJ strain (homozygous for the wild-type Tlr4 allele) was significantly more sensitive to ZnO-induced protein in BAL fluid than the C3H/HeJ strain (homozygous for a mutant Tlr4 allele) after a 1× exposure (Table 1).
Unlike what was seen with acid-coated particle-induced lung inflammation, however, C3H/HeOuJ mice were more susceptible than C3H/HeJ mice to ZnO-induced PMNs in BAL fluid after a single exposure to 1.0 mg/m³ of ZnO. Tlr4 did not appear to confer tolerance to ZnO-induced lung injury after five successive exposures because C3H/HeOuJ mice displayed a tolerant phenotype with regard to PMNs and protein in BAL fluid. It is unclear from our data, however, whether Tlr4 regulates the nontolerant phenotype because of the lack of PMN and protein responses after a single exposure in the C3H/HeJ strain.

Inferences regarding genetic mechanisms can be made by comparing the relative sensitivities to lung injury among inbred strains exposed to O₃ (32-34) or nickel sulfate (57). In general, the C3H/HeJ strain appears to be the most resistant to changes in BAL PMNs and protein from single O₃ (acute, 2 ppm for 3 h; subacute, 0.3 ppm for 48 h), nickel sulfate (150 g of Ni/m³ for 48 h), and ZnO (1.0 mg/m³ for 3 h) exposures. Conversely, the D2 strain appears to be resistant to the adverse pulmonary effects from acute and subacute O₃ exposure but was quite sensitive to both 1× and 5× ZnO exposures. Likewise, C57BL/6J mice were susceptible to O₃- and nickel sulfate-induced PMNs and protein in BAL fluid but were relatively resistant to single and repeated ZnO exposure. Due to differences in the development of tolerance between intermittent and continuous O₃ exposure (55), caution must be taken when interpreting the comparison of 5× ZnO responses with those from subacute O₃ and nickel sulfate exposures. However, the discordance in responses among the inbred mice used in these studies suggests that separate genes may control susceptibility to PMNs and protein in BAL fluid, not only within a given strain, but also between different types of inhaled agonists.

In summary, we have found that significant interstrain variation exists in the acquisition of pulmonary tolerance to repeated ZnO exposure. Thus the development of tolerance has a significant genetic component. CByD2F1/J mice were also examined to determine the mode of inheritance of pulmonary tolerance. Furthermore, comparison of phenotypes between CBy and D2 inbred mice after repeated exposure to O₃ and aerosolized endotoxin suggested that the development of pulmonary tolerance is controlled via multiple molecular mechanisms. This model will be useful in determining candidate genes that play important roles in these mechanisms and may help in the understanding of the human health effects of repeated pollutant exposure.

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