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Endotoxin responsiveness and subchronic grain dust-induced airway disease

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George, Caroline L. S., Hong Jin, Christine L. Wohlford-Lenane, Marsha E. O'Neill, John C. Phipps, Patrick O'Shaughnessy, Joel N. Kline, Peter S. Thorne, and David A. Schwartz. Endotoxin responsiveness and subchronic grain dust-induced airway disease. *Am J Physiol Lung Cell Mol Physiol* 280: L203–L213, 2001.—Endotoxin is one of the principal components of grain dust that causes acute reversible airflow obstruction and airway inflammation. To determine whether endotoxin responsiveness influences the development of chronic grain dust-induced airway disease, physiological and airway inflammation remodeling parameters were evaluated after an 8-wk exposure to corn dust extract (CDE) and again after a 4-wk recovery period in a strain of mice sensitive to (C3H/HeBFeJ) and one resistant to (C3H/HeJ) endotoxin. After the CDE exposure, both strains of mice had equal airway hyperreactivity to a methacholine challenge; however, airway hyperreactivity persisted only in the C3H/HeBFeJ mice after the recovery period. Only the C3H/HeBFeJ mice showed significant inflammation of the lower airway after the 8-wk exposure to CDE. After the recovery period, this inflammatory response completely resolved. Lung stereological measurements indicate that an 8-wk exposure to CDE resulted in persistent expansion of the airway submucosal cross-sectional area only in the C3H/HeBFeJ mice. Collagen type III and an influx of cells into the subepithelial area participated in the expansion of the submucosa. Our findings demonstrate that subchronic inhalation of grain dust extract results in the development of chronic airway disease only in mice sensitive to endotoxin but not in mice that are genetically hyporesponsive to endotoxin, suggesting that endotoxin is important in the development of chronic airway disease.

asthma; airway remodeling; genetics; environmental exposure

OCCUPATIONAL EXPOSURE TO GRAIN dust has been shown to cause lower airway disease characterized by acute changes in airflow and the development of asthma and chronic obstructive lung disease (34, 61). The prevalence of the acute changes in airflow that occur across a work shift (decrease in forced expiratory volume in 1 s by at least 10%) is between 4 and 11% of grain workers (12, 22). Chronic exposure to grain dust can cause irreversible and progressive airway disease. Epidemiological studies performed in North America (23, 61), the United Kingdom (6), Egypt (25), and South Africa (70) demonstrate that workers chronically exposed to grain dust are at increased risk of developing chronic cough, wheeze, and dyspnea irrespective of smoking habits. Long-term follow-up studies have shown that grain workers (13), as well as other workers exposed to organic dusts (17, 27, 59), have accelerated airflow obstruction. Although short-term experimental (20) or occupational (10) exposure to grain dust results in reversible airway symptoms and airflow obstruction, long-term occupational exposure to either grain dust (13, 42) or cotton dust (3) causes irreversible and progressive airway disease. Interestingly, decreases in pulmonary function that occur across a work shift were predictive of continued annual declines in pulmonary function in cotton workers (27), agricultural workers (59), and seasonal grain handlers (52). Although the work shift response to organic dust may simply identify a cohort of individuals with a high intrinsic risk of airway disease, it is equally possible that the acute physiological and biological responses to inhaled organic dusts are involved in the pathogenesis of progressive airway disease.

Several lines of evidence indicate that endotoxin is one of the primary agents in organic dust that cause acute changes in airway physiology and airway inflammation. Previous studies have demonstrated that increasing concentrations of inhaled endotoxin contained in cotton dust are associated with increased airway symptoms and acute decline in pulmonary function among cotton workers (41). Inhaled endotoxin (46), grain dust (18), and cotton dust (11) can all cause airflow obstruction in previously unexposed human subjects. Naive, healthy study subjects challenged with dust from animal confinement buildings develop airflow obstruction and an increase in the serum concentration of neutrophils and interleukin-6 (IL-6), all of

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which are most strongly associated with the concentration of endotoxin (not dust) in the bioaerosol (73). Although the association between the concentration of endotoxin in cotton dust and lung disease in cotton workers is not clear (16, 17), occupational exposure to endotoxin in agricultural workers is associated with a chronic decrement in lung function (52, 60). Finally, our previous exposure-response studies have shown that inhaled grain dust and endotoxin produce similar physiological and biological effects in humans (18, 20) and mice (20, 58). The concentration of endotoxin in grain dust has an important role in the acute biological response to grain dust in humans (37) and mice (58). A competitive antagonist for endotoxin (*Rhodobacter spheroides* diphosphoryl lipid A) reduces the inflammatory response to inhaled grain dust extract in mice (36), whereas removing endotoxin from grain dust by a polycationic nylon filter or with polymyxin B beads renders the extract significantly less proinflammatory (35). Last, genetic or acquired hyporesponsiveness to endotoxin substantially reduces the biological response to grain dust in mice (58). Taken together, these studies indicate that endotoxin is an important cause of acute grain dust-induced airway disease.

To determine the role of endotoxin in the development of chronic grain dust-induced airway disease, we challenged endotoxin-sensitive mice (C3H/HeBFeJ) and mice (C3H/HeJ) hyporesponsive to endotoxin with subchronic exposures of inhaled grain dust. The C3H/HeJ mice are genetically hyporesponsive to endotoxin by virtue of a mutation in the Toll-like receptor-4 (TLR-4) gene, which impedes endotoxin signal transduction in cells (53). We hypothesized that the C3H/HeBFeJ mice, capable of developing an acute inflammatory response to inhaled endotoxin, would develop chronic grain dust-induced airway disease. In contrast, we anticipated that C3H/HeJ mice, unable to respond to inhaled endotoxin, would not develop chronic airway disease when challenged with subchronic doses of grain dust. Our results indicate that subchronic exposure to extracts of grain dust causes a chronic airway process characterized by persistent airway hyperreactivity and airway remodeling in C3H/HeBFeJ but not in C3H/HeJ mice.

METHODS

Overview. To test our hypothesis, we exposed both endotoxin-sensitive (C3H/HeBFeJ) and endotoxin-resistant (C3H/HeJ) mice to corn dust extract (CDE) for an 8-wk period. Our chosen method of delivering the corn dust as an extract has been used in previous studies (26, 27, 41, 47) and provides a more reliable delivery of the corn dust particles and endotoxin concentration than if we were to use a dry aerosol. In addition, it provides better deposition of small particles, which are needed to reach the alveolar and tracheobronchial regions of the murine respiratory tract (33). However, it must be acknowledged that the extract is clearly different from the dry dust that grain workers inhale. Mice were evaluated before exposure to the CDE, immediately after the exposure, and 4 wk after the 8-wk exposure. Physiological responses to the CDE exposure were assessed in a whole body plethysmograph by estimating airway resistance and expressing these changes as the enhanced pause pressure (P_{enh}) during a methacholine challenge. The inflammatory response was analyzed by measuring the concentration of cells and cytokines [tumor necrosis factor-α (TNF-α), IL-6, and macrophage inflammatory protein (MIP-2)] in whole lung lavage fluid and the relative concentrations of TNF-α, IL-6, and MIP-2 mRNAs in lung homogenates. These specific cytokines were chosen because these proteins have been shown to have a role in the acute inflammatory response to inhaled grain dust in humans and mice (20, 68). Evidence for airway remodeling was evaluated by stereology, and different airway wall components were identified by immunohistochemical staining for collagen type III and smooth muscle actin.

Animals. We obtained C3H/HeBFeJ and C3H/HeJ male mice from Jackson Laboratories (Bar Harbor, ME) at 6–8 wk of age. The CDE exposures were then initiated when the mice were 8–10 wk old. The mice were divided into three treatment groups: *1*) those examined before exposure (termed "baseline"), *2*) those exposed to CDE for 4 h/day, 5 days/wk, for 8 wk (termed "8-wk CDE"), and *3*) those exposed to 8-wk CDE and then allowed 4 wk of recovery time in the animal vivarium (termed "recovered"). Age-matched strain-specific controls used in the lung stereology experiments were housed in the animal vivarium until death. All animal care and housing requirements set forth by the National Institutes of Health Committee on Care and Use of Laboratory Animal Resources were followed. All protocols used in this study were approved by the Institutional Animal Care and Use Committee. Mice were provided food (Formulab Chow 5008; Purina Mills, Richmond, IN) and water ad libitum.

CDE preparation and exposure. The corn dust used in these studies was obtained from an air filtration system at a local Iowa grain elevator. The extracts were prepared as previously described (58). Briefly, 3.0 g of dust were combined with 30.0 ml of pyrogen-free saline, vortexed for 2 min, and then agitated for 1 h at 4°C. The suspension was then centrifuged at 2,800 *g* for 30 min to remove insoluble particles with a density greater than water. The resulting supernatant was then filtered through a 0.45-μm low-protein-binding sterile nonpyrogenic polyvinylidene difluoride filter (Acrocap; Gelman Sciences, Ann Arbor, MI) to yield the CDE. This filter has a low affinity for endotoxin yet renders the extract sterile (35, 58). The extract was adjusted to pH 7.0 and stored at −70°C until used. Sterility was confirmed by culture on trypticase soy agar at 35°C and 52°C and malt extract agar at 25°C. The concentration of endotoxin in the CDE was determined by the *Limulus* amebocyte lysate assay (see below).

Immediately before use, the CDE stock was diluted with Hanks' balanced salt solution at a predetermined concentration for nebulization. CDE aerosol was generated and directed into a glass 40-liter exposure chamber by use of a Collison nebulizer (BGI, Waltham, MA). Filtered and dehumidified air was supplied to the nebulizer under 20 psi gauge pressure and at a flow rate of 12 l/min. Mixing within the chamber was aided by a magnetically coupled rotor. The chamber was exhausted at a metered flow of 27.0 l/min. Exposures were quantified as described previously (65). The aerosolized CDE had a mass median aerodynamic diameter of 1.4 ± 1.5 μm, which likely contained other bioaerosol components of grain dust not eliminated by the centrifugation and filtration process (41). Although some of these plant and microbial components may contain glucans, biologically active polyglucose compounds that could contribute to the inflammatory effects of CDE (26, 72), we have found that the concentrations of glucans were not significant enough to influence the inflammatory response to CDE or to interfere

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with the endotoxin measurements (see below). Endotoxin concentrations were evaluated by sampling the chamber outflow filters. The endotoxin concentrations averaged 2.1–2.3 $\mu g/m^3$ over the 8-wk exposure period.

Endotoxin assay. The endotoxin concentrations of the CDE solution and resulting aerosols were assayed using the chromogenic *Limulus* amoebocyte lysate assay (QCL-1000, Whittaker Bioproducts, Walkersville, MD) with sterile pyrogen-free laboratory ware and a temperature-controlled microplate block and microplate reader (405 nm). This technique has been described previously by our laboratory (58). Briefly, the CDE stock solutions were serially diluted in pyrogen-free water and assayed to create the appropriate dilutions. The airborne concentration of endotoxin was assessed by sampling 0.40 m^3 of air drawn from the exposure chamber through 47-mm binder-free glass microfiber filters (Whatman, Clifton, NJ) held within a 47-mm stainless in-line air-sampling filter holder (Gelman Sciences, Ann Arbor, MI). Four separate samples were taken at evenly spaced time intervals during each 4-h exposure, and a daily concentration was determined from each set of four filters. Endotoxin was measured from the exposure chamber outflow filters by extracting the endotoxin with 10 ml of pyrogen-free water at room temperature with gentle shaking for 1 h. The extracts were then serially diluted and assayed for endotoxin. All standard curves achieved a linear regression coefficient exceeding $r = 0.995$. Endotoxin concentrations expressed as endotoxin units (EU) were converted to mass units as follows: 10 EU/ng for the EC-5 US reference standard endotoxin. Addition of β-glucan blocker (Whittaker Bioproducts) to duplicate *Limulus* amebocyte lysate assay samples demonstrated that glucans from either fungal or plant materials were not present in large enough concentrations to interfere with our endotoxin measurements.

Airway physiology. Airway resistance was estimated during a methacholine challenge. Individual mice ($n = 10–12$ mice per condition) were placed in an 80-ml whole body plethysmograph (Buxco Electronics, Troy, NY) that was ventilated by bias airflow at 0.2 l/min. This unit was interfaced with differential pressure transducers, analog-to-digital converters, and a computer. The breathing patterns and pulmonary functions of each individual mouse were monitored over time. Direct measurements were made of the respiratory rate, pressure change within the plethysmograph, and "box flow," which is the difference between the animal's nasal airflow and the flow induced by thoracic movement; this difference varies in the presence of airflow obstruction because of pulmonary compression (due to forced expirations). The Buxco system measured both the magnitude of the box pressure variations and the slope of the box pressure; associated software also evaluated the wave shape, which is most dramatically changed during early expiration. Airway resistance was estimated by exposing mice to increasing doses of methacholine and recording the P_{enh}. P_{enh} = (expiratory time/40% of relaxation time − 1) × peak expiratory flow/peak inspiratory flow × 0.67. The validity of P_{enh} as an estimation of bronchoconstriction has been examined (28). Lung function was evaluated at baseline and after stimulation with inhaled methacholine (12.5 and 25 mg/ml) according to a standard protocol (44).

Lung lavage and lung preparation. Mice were killed by cervical dislocation. The trachea was exposed, and lungs were lavaged through a PE-90 tube with 6.0 ml of sterile saline, 1 ml at a time, at a pressure of 25 cmH_2O. Return volume was recorded and was consistently greater than 4.5 ml. The lungs were then removed and snap-frozen in liquid nitrogen and stored at −70°C for further use.

Processing of the lavage fluid has been described previously (58). Briefly, the lavage fluid was centrifuged for 5 min at 200 *g*. The supernatant was decanted and stored at −70°C for further use. The cell pellet was resuspended with Hanks' balanced salt solution (without Ca or Mg) and washed twice. A small aliquot of resuspended cells was used for counting cells using a hemocytometer. The cells were washed once more and resuspended in RPMI medium so that the final suspension had a final concentration of 1×10^6 cells/ml. Ten microliters of the cell suspension was spun onto a slide using a cytocentrifuge (Shanden, Southern Sewickley, PA). Postcytospin cells were stained with Diff-Quik Stain Set (Harleco, Gibbstown, NY), air-dried, and covered with a coverslip with Permount (Fisher, Pittsburgh, PA).

Cytokine evaluation. TNF-α and IL-6 were measured by ELISA using capture and biotinylated detection antibodies specific for murine TNF-α and IL-6 from Genzyme (Cambridge, MA) and PharMingen (San Diego, CA), respectively. Detection was increased by the addition of avidin-horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) before development with the chromagen tetramethylbenzidine (Sigma, St. Louis, MO). The reaction was stopped with the addition of 100 μl/well of 0.67 N H_2SO_4. The murine MIP-2 ELISA kit was purchased from R&D Systems (Minneapolis, MN). One hundred microliters of whole lung lavage fluid were run in duplicate for each assay. Standard curves were run with each ELISA. The lower limit of detection for each protein was as follows: 5.1 pg/ml for TNF-α, 10 pg/ml for IL-6, and 1.5 pg/ml for MIP-2.

Preparation of RNA and multiprobe RNase protection assay. Total RNA was extracted from lung specimens using the single-step method (14, 40), lysing flash-frozen lung in RNA STAT-60 (Tel-Test B, Friendswood, TX). The composition of RNA STAT-60 includes phenol and guanidinium thiocyanate in a monophase solution. The lung tissue was homogenized in the RNA STAT-60 using a polytron homogenizer. Chloroform was added, and the total RNA was precipitated from the aqueous phase by addition of isopropanol. The total RNA was washed with ethanol and solubilized in RNase-free water. The yield and purity of RNA were quantified by measuring the ratio of absorbances at 260 and 280 nm. Minigel electrophoresis was used to confirm the integrity of the 28S and 18S rRNA bands. Gene transcripts were detected using the RNA and probes as previously described (30). Ten micrograms of total RNA were hybridized with a ^{32}P-labeled antisense cRNA probe in a hybridization buffer solution for 14 h at 56°C. The nonhybridized single-strand RNA was digested with a mixture of RNases A and T1. The remaining protected RNA fragment was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and then ethanol precipitated. The protected hybridization products were separated on a 5% acrylamide-8 M urea gel. The gel was dried on a vacuum gel dryer at 80°C, wrapped in plastic wrap, and exposed to X-ray film for 12 h at −70°C.

Immunohistochemistry and stereology. C3H/HeBFeJ and C3H/HeJ mice, $n = 6–8$ per condition, were used for obtaining lung tissue for immunohistochemistry and stereology measurements. Lungs were excised and infused with 10% buffered Formalin to a pressure of 25 cmH_2O as has been described previously for stereology (64). Lungs were sliced in the sagittal plane, with the midsagittal section used for immunohistochemical and stereological analysis. Tissue blocks were processed through a graded ethanol series and embedded in paraffin. Lung tissue from age-matched controls for each strain of mice was obtained in the same manner for lung stereology.

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Lung sections used for immunohistochemical staining underwent deparaffinization and hydration with xylenes, ethanols, and water. Pretreatment with 0.01 M sodium citrate, pH 6.0, for 10 min at 95°C followed by 1 mg/ml pepsin in 0.05 M acetic acid for 2 h at 37°C was required for detection of collagen type III. All slides were washed in Tris-buffered saline, pH 7.5, before application of normal serum from the animal the secondary antibody was raised in (goat or rabbit serum) for blocking. For detection of collagen type III, a rabbit anti-human antibody was used (Biogenesis, Sandown, NH) at 1:100 dilution, and for actin, a rabbit anti-chicken smooth muscle actin antibody (Biogenesis) was used at 1:500. All primary antibodies were diluted in the respective blocking serum. Incubation times were at least 2 h to overnight at 4°C. Actin was detected with a Vectastain Elite peroxide kit (Vector, Burlingame, CA) and developed with the chromagen 3,3′-diaminobenzidine (Sigma, St. Louis, MO). The slides were counterstained with 0.5% methyl green, washed in water and butanol before xylenes, and covered with a coverslip with Permount. The collagen type III was processed with a Vectastain anti-goat IgG ABC-AP kit and developed with Vector Red chromagen before being counterstained with hematoxylin, and then dehydrated with ethanols and xylenes. Slides were covered with a coverslip with Permount.

Stereology was performed using standard methods developed by Cruz-Orive and Weibel (19) and Hogg et al. (31). Sections 8 μm thick were cut and stained with hematoxylin and eosin. Airway perimeters and wall areas were examined by capturing all conducting airway images at ×20 with a Spot Jr. digital camera (Diagnostic Instruments, Sterling, MI) and analyzed using Image-Pro Plus computer software. Slides were coded and measured by two observers blinded to the codes. Measurements used in the study have been previously described (31) and are illustrated in Fig. 1. These include internal perimeter, external perimeter, and basement membrane perimeter. Areas calculated using these measurements include the submucosal and epithelial areas. All airways from the experimental and age-matched control mice were divided into three relatively equal groups by their airway width. This permitted us to examine the effect of inhaled grain dust on "small airways" (0–90 μm), "medium airways" (>90–129 μm), and "large airways" (>129 μm). Any airway cut obliquely and showing a length-to-width ratio greater than 2.5 was not used for analysis.

Statistical analysis. Comparisons were performed to investigate the difference between the C3H/HeBFeJ and C3H/HeJ mice in airway physiology and the inflammatory response to inhaled CDE. Airway physiology was compared between the C3H/HeBFeJ and the C3H/HeJ mice by analyzing the ratio of P_{enh} at a given methacholine dose to P_{enh} at 0 mg/ml methacholine. At each time point, the concentration of whole lung lavage cells, cytokines, and mRNAs and the areas of the epithelia and submucosa were compared between the two strains of mice. The Mann-Whitney *U*-test was used to assess statistical significance for these comparisons (56). For the stereology measurements, intraobserver reproducibility was assessed using the coefficient of variation, comparing at least 10% of airways in each experimental group between the two observers. Reliability coefficients were 0.98 or greater.

RESULTS

Airway resistance. Before the exposure, there were no significant differences in the estimated measurements of airway hyperreactivity between the two strains of mice (Fig. 2*A*, baseline C3H/HeBFeJ and C3H/HeJ). Within 24 h after the subchronic CDE inhalation challenge, both the C3H/HeBFeJ mice and the C3H/HeJ mice demonstrated significant airway hyperreactivity (Fig. 2*A*, 8-wk CDE C3H/HeBFeJ and C3H/HeJ). Yet after the 4-wk recovery period, only the C3H/HeBFeJ mice continued to demonstrate significant ($P < 0.05$) airway hyperreactivity to the methacholine challenge. (Fig. 2*B*).

Inflammation in the lower respiratory tract. At baseline and after the recovery period, the concentration of cells and cytokines in the whole lung lavage fluid was similar in the C3H/HeBFeJ mice and the C3H/HeJ mice (data not presented). However, after the 8-wk inhalation exposure to CDE, the C3H/HeBFeJ mice demonstrated a profound inflammatory response, whereas the C3H/HeJ mice did not have evidence of inflammation in the lower respiratory tract (Figs. 3 and 4). Specifically, immediately after the 8-wk exposure, the C3H/HeBFeJ mice had a significant ($P < 0.05$) increase in the total number of cells, percent

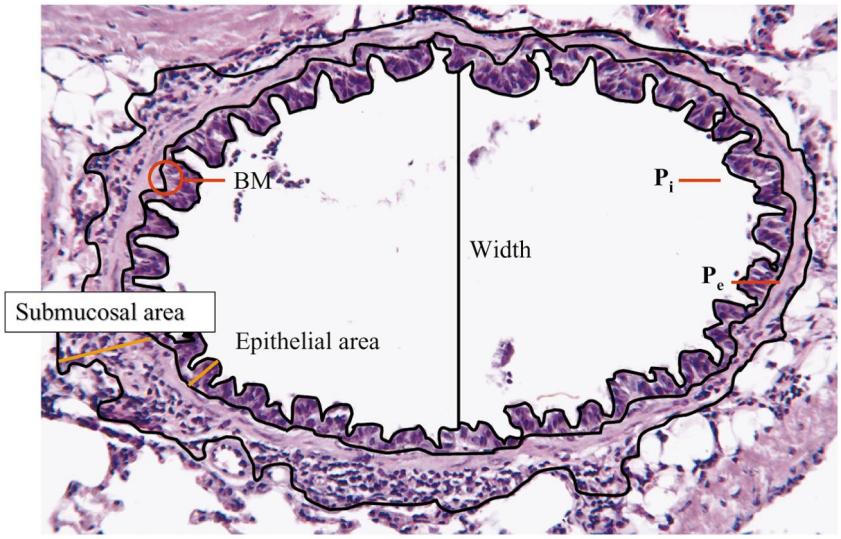

Fig. 1. Stereological measurements were obtained using standard methods as developed by Cruz-Orive and Weibel (19) and Hogg et al. (31). Sections 8 μm thick were cut and stained with hematoxylin and eosin. Airway perimeters and wall areas were traced using Image-Pro Plus computer software. Measurements (red lines) included length, width, internal perimeter (P_i), external perimeter (P_e), and basement membrane perimeter (BM). Areas calculated were the epithelial area and submucosal area. All airways were evaluated on each microscope slide.

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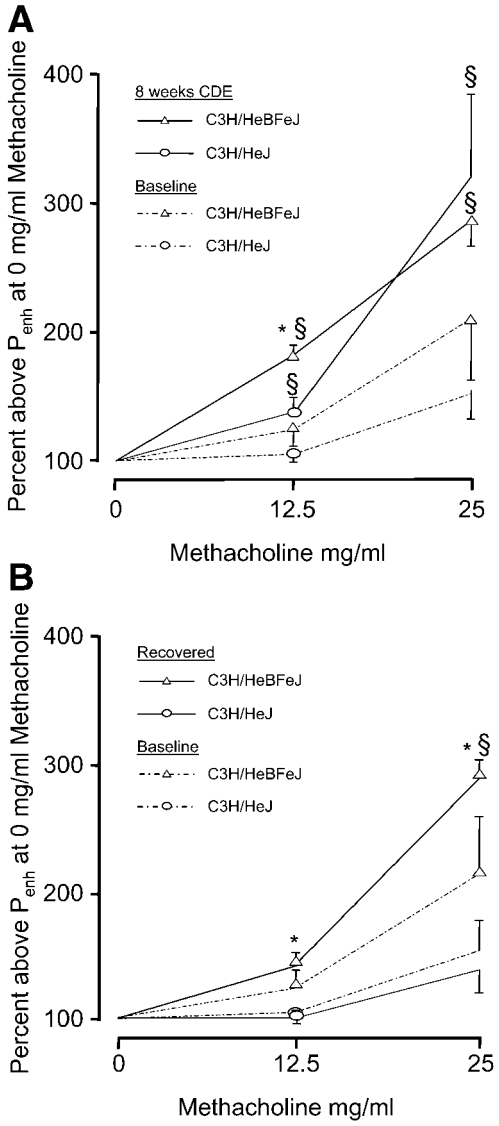

Fig. 2. Airway hyperreactivity is expressed as enhanced pause pressure (P_{enh}) ratio (P_{enh} at a given methacholine concentration to P_{enh} at 0 mg/ml methacholine) immediately after 8-wk inhalation of corn dust extract (CDE; *A*) and after the 4-wk recovery period (*B*) in C3H/HeJ (solid lines, ○) and C3H/HeBFeJ (solid lines, △) mice. P_{enh} was measured after each increasing dose of methacholine as described in METHODS. Baseline data from the same animals before the start of the CDE exposure in C3H/HeJ mice (dashed lines, ○) and C3H/HeBFeJ mice (dashed lines, △) are also presented. Error bars are the SE. $*P < 0.05$ comparing C3H/HeBFeJ and C3H/HeJ animals exposed to CDE. $\S P < 0.05$ comparing data from CDE-exposed animals to their strain-specific preexposure data.

neutrophils, and TNF-α, IL-6, and MIP-2 proteins in their lavage fluid compared with the C3H/HeJ mice (Figs. 3 and 4). Immediately after the end of the 8-wk inhalation challenge, the C3H/HeBFeJ mice demonstrated increases in TNF-α and IL-6 mRNA levels, an effect that was not observed in the C3H/HeJ mice (Fig. 5). Interestingly, an increase in MIP-2 mRNA production was increased in both C3H/HeBFeJ and C3H/HeJ mice immediately after the 8-wk CDE inhalation exposure; however, the production was much more pronounced in the endotoxin-sensitive strain.

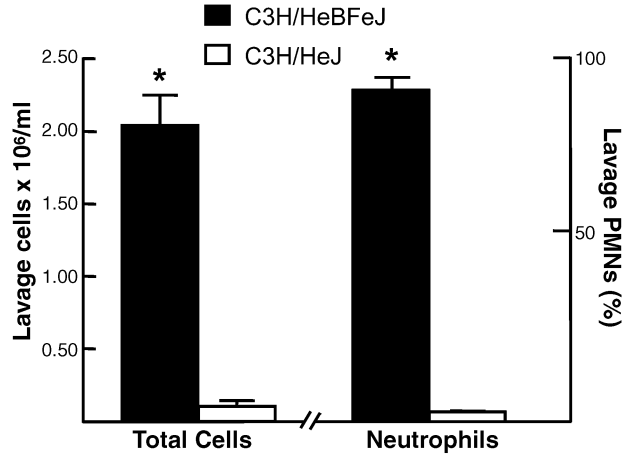

Fig. 3. Lung lavage fluid mean concentration of total cells and percentage of neutrophils immediately after 8-wk inhalation of grain dust extract in C3H/HeJ (open bars) and C3H/HeBFeJ (solid bars) mice. PMNs, polymorphonuclear cells. Error bars are SE. $*P < 0.05$.

Airway architecture. After the subchronic inhalation of CDE, C3H/HeBFeJ, but not C3H/HeJ, mice demonstrated thickened airway walls, specifically in the airway submucosa (Fig. 6). In airways of all sizes, the C3H/HeBFeJ mice demonstrated significantly ($P < 0.05$) larger submucosal cross-sectional areas in their conducting airways compared with the C3H/HeJ mice, as well as with their strain-specific age-matched controls, immediately after the CDE exposure. After the 4-wk recovery period, the C3H/HeBFeJ mice continued to have significantly ($P < 0.05$) larger submucosal areas compared with their age-matched controls. The C3H/HeJ mice varied significantly ($P < 0.05$) from their age-matched controls only in the large airways after the 4-wk recovery period (Fig. 6*C*). The epithelial cross-sectional area of conducting airways did not vary over time or between strains of mice (data not presented). Only the epithelial cross-sectional area of

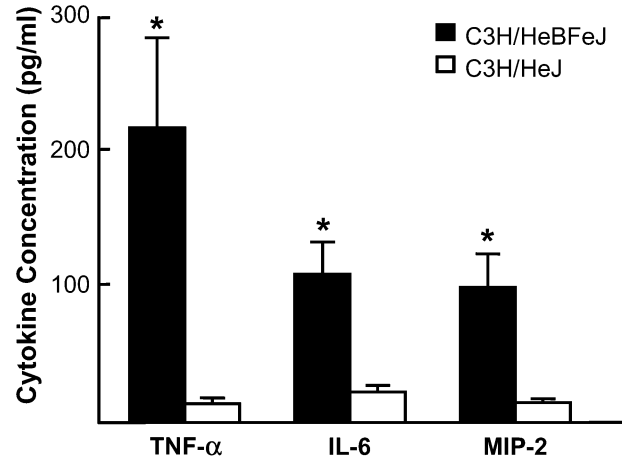

Fig. 4. Lung lavage fluid mean concentration of tumor necrosis factor-α (TNF-α), macrophage inflammatory protein (MIP-2), and interleukin-6 (IL-6) protein immediately after 8-wk inhalation of grain dust extract in C3H/HeJ (open bars) and C3H/HeBFeJ (solid bars) mice. Error bars are SE. $*P < 0.05$.

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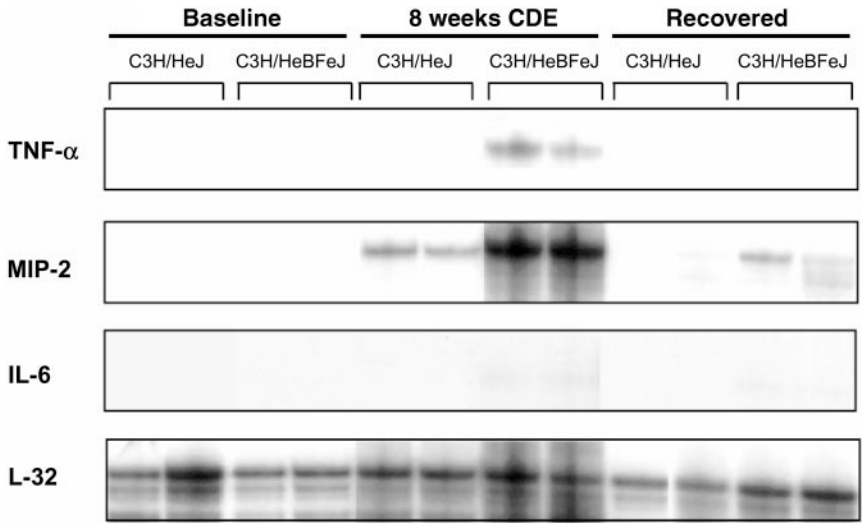

Fig. 5. RNase protection assay of total RNA obtained from lungs before (baseline), immediately postexposure (8-wk CDE), and after the 4-wk recovery period (recovered) in C3H/HeJ and C3H/HeBFeJ mice. Equivalent amounts of RNA were examined in each sample as judged by the amount of L-32, which encodes a ubiquitously expressed ribosome subunit protein.

large conducting airways from the C3H/HeBFeJ mice was significantly greater ($P < 0.05$) immediately after the 8-wk CDE exposure compared with their age-matched controls (Fig. 7). No changes were observed in the length of the basement membrane, indicating that the airway size between the two species of mice was similar and did not change over time (data not shown).

Immunohistochemical staining indicated that actin was present in small, elongated cells arranged circumferentially in the airway submucosa, whereas collagen type III stained positive in a thin circumferential line in the submucosa (Figs. 8 and 9). Figure 8 demonstrates the patchy actin staining in a C3H/HeBFeJ mouse exposed to 8 wk of inhaled CDE. The staining for actin in this photomicrograph is representative of what was observed in both strains of mice at all time points. After an 8-wk CDE exposure, both the C3H/HeBFeJ and C3H/HeJ mice had similar collagen type III deposition (Fig. 9, 8-wk CDE exposure). After the 4-wk recovery period, the C3H/HeBFeJ mice demonstrated increased staining for collagen type III, whereas the C3H/HeJ mice were observed to have a thin rim of collagen deposition around the airway (Fig. 9, recovered mice).

DISCUSSION

Our results demonstrate that subchronic inhalation of grain dust extract results in the development of airway disease in mice sensitive to endotoxin but not in mice genetically hyporesponsive to endotoxin. These results suggest that endotoxin, or at least signaling via the TLR-4 pathway, is critical for the development of grain dust-induced lung injury. The airway disease observed in this murine model of subchronic inhalation of grain dust extract is similar to the airway disease observed in workers chronically exposed to grain dust and other forms of organic dusts. Specifically, there is the development and persistence of airway hyperreactivity, an acute lung inflammatory response, and airway remodeling (18, 27). We acknowledge that during this subchronic exposure to CDE, the animals were likely exposed to nebulized particles smaller than 0.45 μm. Recent literature supports the idea that particulate matter in our environment is an etiology of lung injury; however, Oberdörster et al. (51) have shown that it is particulate matter smaller that 20 nm that is most strongly associated with lung injury. Importantly, the chronic loss of airflow among agriculture workers (59) and grain handlers (61) is significantly associated with the concentration of inhaled endotoxin in the work place. Likewise, our animal model supports the importance of endotoxin in this type of lung injury. Therefore, our model of chronic airway disease in mice exposed to grain dust extract may prove useful in understanding the biology of organic dust-induced airway disease as well as of other obstructive airway diseases.

Endotoxin may have a larger role in chronic airway disease than previously realized. Recent reports have indicated that the concentration of endotoxin in the domestic setting is related to the clinical severity of asthma (47, 48). Moreover, asthmatic individuals develop airflow obstruction at lower concentrations of inhaled endotoxin (46), and inhalation of allergens increases the ability of the lung to respond to endotoxin (24). Interestingly, inhaled allergens appear to increase the concentration of lipopolysaccharide (LPS) binding protein, a change that allows lung inflammatory cells to respond to the very low concentrations of endotoxin that are commonly present in the airways of uninfected lungs (24). In addition, endotoxin may have a role in airway disease caused by air pollution as well as by other occupational exposures such as the manufacture of fiberglass (4, 7, 49). Recent studies have shown that particulate matter, which is strongly associated with airway disease (21), is contaminated with endotoxin (4, 7). Moreover, the concentration of endotoxin in particulate matter is directly related to the induction of growth factors (7) and the release of IL-6 and TNF-α (4) by alveolar macrophages. These findings suggest that endotoxin may contribute to the development of lung diseases other than occupational

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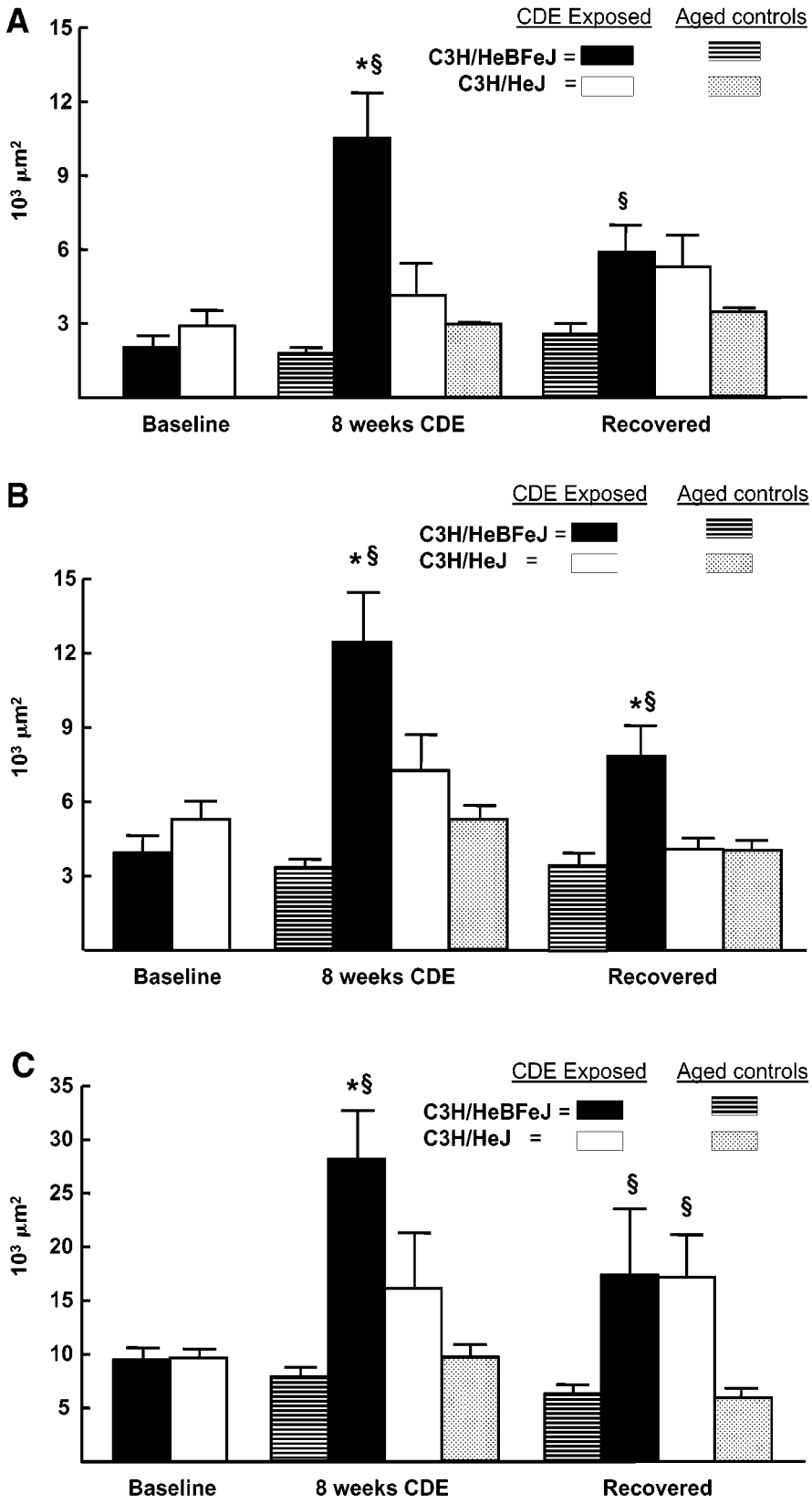

Fig. 6. Stereological measures of the submucosal area from small (*A*), medium (*B*), and large (*C*) airways from C3H/HeBFeJ mice (solid bars) and C3H/HeJ mice (open bars) exposed to CDE, as well as unexposed age-matched control C3H/HeBFeJ mice (striped bars) and C3H/HeJ mice (speckled bars) are presented at baseline, after 8-wk CDE exposure, and after the 4-wk recovery period. Error bars are the SE. $*P < 0.05$ for exposed C3H/HeBFeJ vs. C3H/HeJ mice. $\S P < 0.05$ for CDE-exposed mice vs. strain-specific age-matched controls.

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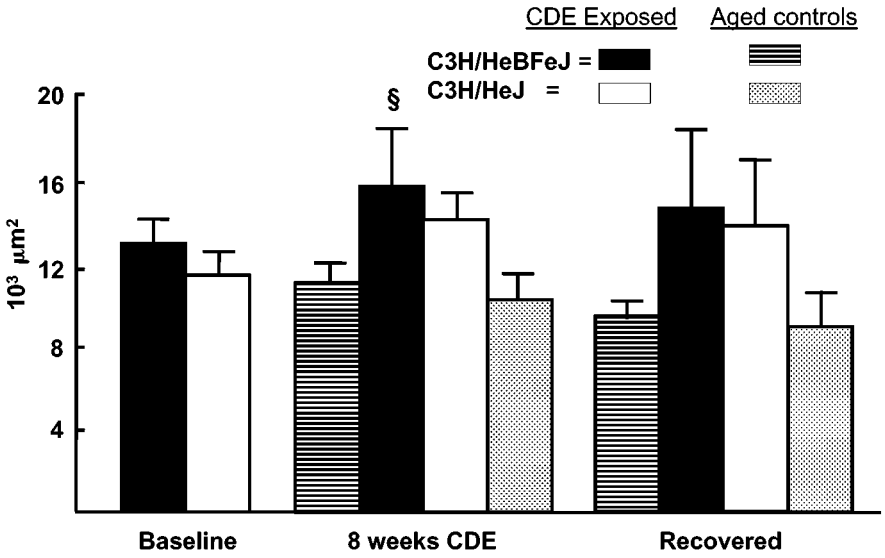

Fig. 7. Stereological measurements of the epithelial area within air spaces from large conducting airways in C3H/HeBFeJ mice (solid bars) and C3H/HeJ mice (open bars) exposed to CDE as well as unexposed age-matched control C3H/HeBFeJ mice (striped bars) and C3H/HeJ mice (speckled bars) are presented at baseline, after 8-wk CDE exposure, and after the 4-wk recovery period. §$P < 0.05$ for CDE-exposed mice vs. strain-specific age-matched controls.

exposures to organic dusts, specifically the development of allergen-induced asthma and air pollution-induced airway disease.

Endotoxin is thought to initiate an inflammatory response by pattern-recognition receptors or proteins (69). The Toll-like receptor family of transmembrane molecules activate proinflammatory transcriptional factors [activator protein-1 and nuclear factor-κB (NF-κB)] after stimulation with endotoxin (45, 69). Even though both TLR-2 and TLR-4 have been implicated in endotoxin signaling (43, 53, 71), TLR-2 does not appear to be essential for endotoxin signaling (29). In fact, whereas TLR-2 knockout mice respond normally to LPS (63), TLR-4 knockout mice do not respond normally to endotoxin (32). Additionally, CD14, a protein known to assist in endotoxin signaling, actually enhances the response of TLR-4 to endotoxin (15). It has been postulated that TLR-4 needs to complex with other proteins to be functional (15, 69). In order for TLR-4 to be functional in mice, a complex formed with the molecule MD-2 is required for endotoxin responsiveness (1, 39). It is specifically the TLR-4 gene that is mutated in the C3H/HeJ mice and is thought to be the cause of the hyporesponsiveness to endotoxin in this strain (38). Interestingly taxol, a compound with a structure unlike that of endotoxin and present in maize roots (2), can activate NF-κB via the TLR-4-MD-2 receptor (39). Taken collectively, these data suggest that C3H/HeJ mice are useful investigational tools with regard to endotoxin signaling or at the very least to the inflammatory response that results from signal transduction via TLR-4.

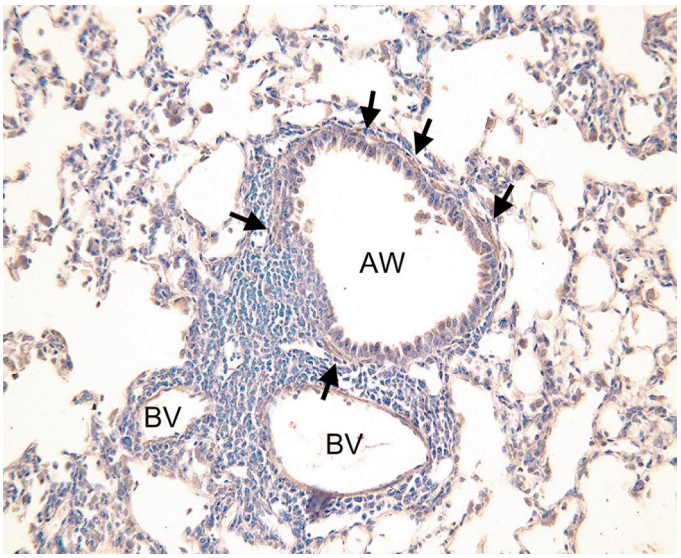

Fig. 8. Smooth muscle actin was identified with a Vectastain anti-rabbit IgG ABC Elite kit, developed with 3-3′-diaminobenzidine before being counterstained with methyl green and dehydration with butanol and xylenes. This photomicrograph is from a C3H/BFeJ mouse exposed to 8-wk CDE and demonstrates patchy staining around the airway in elongated cells just below the epithelial cells (arrows), whereas there is continuous staining around the adjacent blood vessel. It is typical of what was seen in both strains of mice at all time points. AW, airway; BV, blood vessel.

The response that different immunomodulatory cells from C3H/HeJ mice have to endotoxin may vary. Ryan and Vermeulen (57) have demonstrated that alveolar macrophages, and not peritoneal macrophages, from C3H/HeJ mice respond to endotoxin with an increased TNF-α production. A "leaky" endotoxin phenotype in C3H/HeJ mice may explain some of our findings. In vitro alveolar macrophages from C3H/HeJ mice can be induced to respond to LPS (57), and pretreatment with interferon-γ can induce peritoneal macrophages from C3H/HeJ mice to respond normally to endotoxin (5). The C3H/HeJ mice in our studies demonstrated airway hyperreactivity and a slight increase in MIP-2 mRNA immediately after the CDE exposure. These responses occurred in the absence of any change in lung lavage cellularity or cytokine protein alterations. Airway hyperreactivity and inflammation likely coexist in many animal models, but it appears that inflammation is not necessary for hyperreactivity to occur (54, 62, 66). Whether these responses we observed were due to

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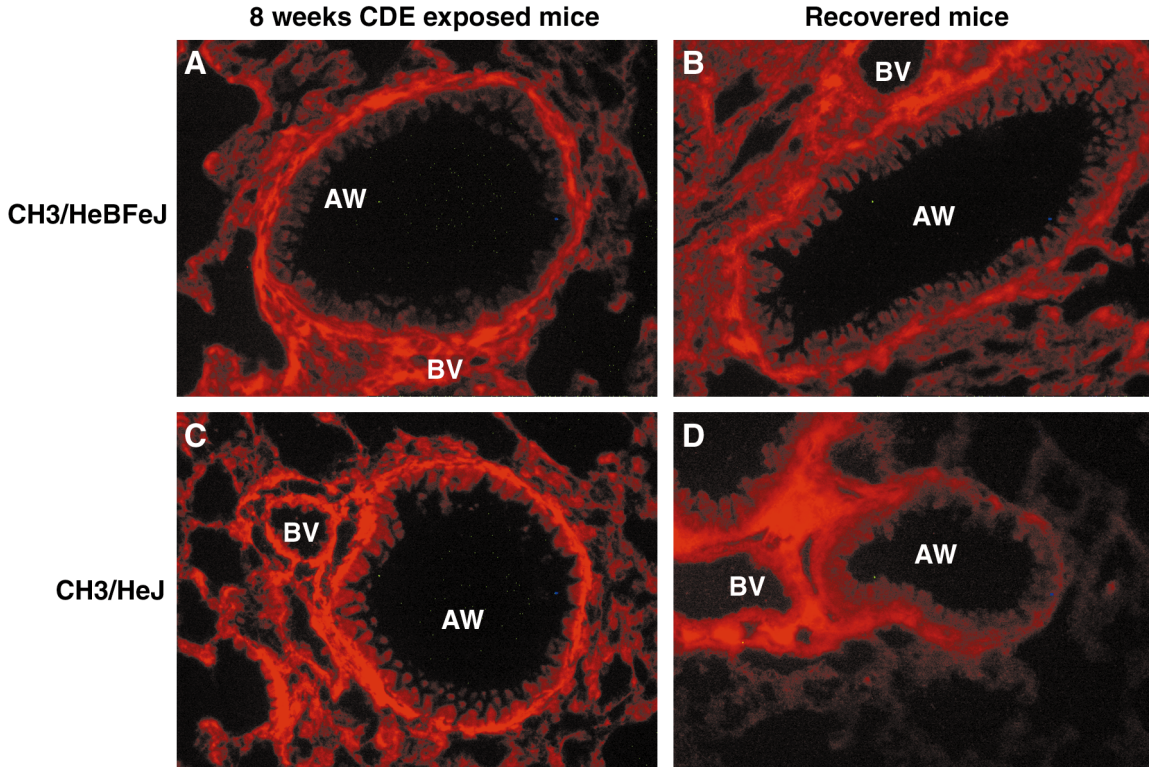

Fig. 9. Type III collagen was identified with a Vectastain anti-goat IgG ABC-AP kit, developed with Vector Red chromagen before being counterstained with hematoxylin and dehydration with ethanols and xylenes. The type III collagen is visualized as intense red staining. Results are presented in these photomicrographs for C3H/HeBFeJ and C3H/HeJ mice immediately after 8-wk CDE exposure (*A* and *C*) and after 4-wk recovery period (*B* and *D*).

alveolar macrophages responding to endotoxin or possibly some other component in the CDE is not entirely clear but suggests that C3H/HeJ mice are responding, albeit minimally, to the subchronic inhalation challenge. Ultimately, the type of inflammatory changes seen only in the airway and interstitium of the C3H/HeBFeJ mice are likely important for the development of the chronic airway changes and persistent airway hyperreactivity.

In the work presented, we have shown that mice exposed to grain dust for as little as 8 wk develop persistent airway hyperreactivity and airway remodeling. Importantly, the airway remodeling in our murine model is characterized by enhanced deposition of collagen III in the subepithelial area, the region directly beneath the airway basement membrane. Symptomatic agricultural workers (60) and asthmatics (38, 55) also have airway hyperreactivity and thickening of the subepithelial region beneath the basement membrane. In asthmatics, this histological feature appears to be directly related to the clinical severity of this disease (50) and airway hyperreactivity to a methacholine challenge (8). Immunohistochemical staining indicates that in asthma, the subepithelial fibrosis predominantly consists of type III collagen as well as type V and fibronectin, which probably originate from fibroblasts and myofibroblasts (9, 55, 67).

The inflammatory response we observed immediately after the subchronic (8-wk) inhalation exposure to grain dust is very similar to that seen after an acute (4-h) exposure in mice (58, 68) and in humans (20). Specifically there is an increase in lung lavage cellularity composed almost entirely of neutrophils and an increase in lung lavage proinflammatory cytokine concentrations. Unique to the subchronic inhalation exposure to grain dust is the presence of mononuclear-appearing cells in the subepithelial area of conducting airways. The presence of these cells in the subepithelial area was not observed after an acute exposure to inhaled grain dust (68). The neutrophil and subepithelial inflammatory responses appear to be important in mediating the development of chronic airway disease given that only the endotoxin-sensitive mice demonstrated both airway inflammation and airway remodeling. The manifestations of airway disease and remodeling persisted in these mice after the neutrophilic and subepithelial inflammatory responses have resolved. Interestingly, immunohistochemical staining of the subepithelial area of asthmatic lungs shows primarily lymphocytes as well as some neutrophils and eosinophils (38). This may be similar to what we have observed in our animal model.

Our results suggest that the neutrophilic inflammatory response and the subepithelial cellular response to inhaled grain dust appear to have important roles in the development of chronic grain dust-induced airway disease. The specific nature of these cell types and their relationship to airway hyperreactivity and chronic disease in this animal model require further investigation. Our airway physiological findings are supported

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by several epidemiological studies that have shown that the acute work shift-related declines in airflow are independently associated with accelerated longitudinal declines in lung function among grain handlers (13) and cotton workers (27). Although the work shift response to organic dust may simply identify a cohort of individuals with a high intrinsic risk of airway disease, it is equally possible that the acute physiological and biological responses to inhaled organic dusts are involved in the pathogenesis of progressive airway disease. This latter hypothesis is supported by findings from this investigation. The inflammatory response and subepithelial airway changes that accompany the persistence of airway hyperreactivity, similar to that seen in asthmatics, also make this animal model of obstructive lung disease potentially applicable to the investigation of airway remodeling in humans.

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