Subchronic endotoxin inhalation causes persistent airway disease

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Brass, D. M., J. D. Savov, S. H. Gavett, N. Haykal-Coates, and D. A. Schwartz. Subchronic endotoxin inhalation causes persistent airway disease. Am J Physiol Lung Cell Mol Physiol 285: L755–L761, 2003. First published June 6, 2003; 10.1152/ajplung.00001.2003.—The endotoxin component of organic dusts causes acute reversible airflow obstruction and airway inflammation. To test the hypothesis that endotoxin alone causes airway remodeling, we have compared the response of two inbred mouse strains to subchronic endotoxin exposure. Physiological and biological parameters were evaluated after 1 day, 5 days, or 8 wk of exposure to endotoxin [lipopolysaccharide (LPS)] in endotoxin-sensitive (C3HeB/FeJ) and endotoxin-resistant (C3H/HeJ) mice. After 5 days or 8 wk of LPS exposure, only C3HeB/FeJ had elevated airway hyperreactivity to inhaled methacholine. Only the C3HeB/FeJ mice had significant inflammation of the lower respiratory tract after 1 day, 5 days, or 8 wk of LPS exposure. Stereological measurements of small, medium, and large airways indicated that an 8-wk exposure to LPS resulted in expansion of the submucosal area only in the C3HeB/FeJ mice. Cell proliferation as measured by bromodeoxyuridine incorporation contributed to the expansion of the submucosa and was only significantly elevated in C3HeB/FeJ mice actively exposed to LPS. C3HeB/FeJ mice had significantly elevated levels of interleukin-1β protein in whole lung lavage after 1 day and 5 days of LPS exposure and significantly elevated protein levels of total and active transforming growth factor-β1 in whole lung lavage fluid after 5 days of LPS exposure. Our findings demonstrate that subchronic inhalation of LPS results in the development of persistent airway disease in endotoxin-responsive mice.

airway remodeling; neutrophilic inflammation; cytokines

INHALATION OF ORGANIC DUSTS containing endotoxin, or inhalation of endotoxin alone, causes neutrophilic inflammation and increased airway resistance in both humans and in animal models (14, 18, 19). Inhaled endotoxin or lipopolysaccharide (LPS) is a risk factor for airway disease (19) and has been shown to be nearly ubiquitous in the environment. In addition to being present in bioaerosols sampled in a variety of agricultural and industrial settings (2, 8, 17, 28), endotoxin may also be a significant component of particulate matter < 10 μm in diameter (29) and may contribute to asthma exacerbation by polluted air (19, 20). Endotoxin is known to be one of the primary agents in organic dusts that cause airway inflammation and acute changes in airway physiology (11).

Subchronic inhalation of grain dust causes airway remodeling characterized by a significant increase in the thickness of the submucosal region of small and medium airways in mice (7). Experimental evidence is emerging to support the view that airway remodeling, caused by subchronic grain dust inhalation, may be mediated by cytokines and growth factors (27). We have previously demonstrated that protein levels of a number of potentially fibrogenic growth factors and cytokines are increased after a single endotoxin inhalation challenge (30). We have also shown that endotoxin is a critical component in grain dust-induced airflow obstruction and airway inflammation (11, 12). The ability of endotoxin alone to recapitulate all the effects of subchronic grain dust inhalation has yet to be established.

Among fibrogenic growth factors and cytokines, several lines of evidence suggest that transforming growth factor (TGF)-β1 and IL-1β may be involved in the airway response to subchronic inhalation of grain dust or endotoxin. TGF-β is most prominent among growth factors with the potential to modulate the extracellular matrix (21), and TGF-β1 can cause differentiation of mesenchymal cells into myofibroblasts (25). These specialized cells have the capacity to secrete extracellular matrix components and also have a contractile phenotype (33). IL-1β increases the expression of TGF-β after intratracheal delivery by an adenoviral vector to mice (16). This increased TGF-β expression persists even after IL-1β levels have returned to baseline (16), suggesting that multiple growth factors and cytokines interact in an expression cascade that could ultimately lead to airway remodeling.

To test the hypothesis that endotoxin alone causes airway remodeling and persistent airway disease, we have compared the response of two inbred mouse strains to subchronic endotoxin exposure. One strain (C3HeB/FeJ) is susceptible to inhaled endotoxin, and the other (C3H/HeJ) is resistant to its effects by virtue
of a naturally occurring mutation in the TLR4 gene that encodes the cell surface receptor for endotoxin. We demonstrate here that, compared with endotoxin-resistant C3H/HeJ mice, endotoxin-sensitive C3HeB/FeJ mice 1) develop an expanded submucosa after 2 mo of repeated endotoxin exposure; 2) develop persistent increased airway resistance after 2 mo of endotoxin exposure; 3) release significant amounts of the fibrogenic growth factor TGF-β and the proinflammatory and potentially fibrogenic cytokine IL-1β into lung lavage fluid; and 4) have elevated rates of cell proliferation in airway epithelium and submucosa.

MATERIALS AND METHODS

Animals. Thirty-two male C3HeB/FeJ and 32 male C3H/HeJ mice from Jackson Laboratories (Bar Harbor, ME) at 8 wk of age were used in this study. Twenty mice of each strain were used for the 8-wk exposure period. Ten were exposed to endotoxin, and 10 were exposed to PBS alone. An additional six mice of each strain were used in the 1-day exposure, and six more mice of each strain were used in the 5-day endotoxin exposure. At the 1- and 5-day time points, mice were killed immediately after plethysmography. Mice exposed to endotoxin for 8 wk were killed 96 h after the end of the last exposure. Mice were injected with 100 mg/kg of 5-toxin for 8 wk were killed 96 h after the end of the last exposure immediately after plethysmography. Mice exposed to endotoxin for 8 wk were killed 96 h after the end of the last exposure. Mice were injected with 100 mg/kg of 5′-bromodeoxy-2′-uridine (BrdU) (Sigma Chemical; St. Louis, MO) sterile filtered in PBS 8 h before death. Whole body plethysmography (described below) was performed on all mice in each group before the beginning of the exposure and again immediately after the 1- and 5-day exposures. Pulmonary function was measured 72 h after the end of the last exposure in the group of animals that was exposed for 8 wk. The study protocol was in accordance with guidelines set forth by both the United States Environmental Protection Agency and the Duke University Animal Care and Use Committee.

LPS preparation and aerosol exposures. LPS was purchased as purified lyophilized powder [lot 110K4060; 25 mg; 30,000,000 endotoxin units (EU)/mg] prepared by phenol extraction from Escherichia coli serotype 0111:B4 from Sigma Chemical. LPS was reconstituted with 10 ml of sterile PBS, and stock aliquots (2.5 mg/ml) were stored at −20°C. Stock aliquots were thawed once and stored at 4°C thereafter. Immediately before use, 60 μl of LPS stock (1.75 mg; 2,625,000 EU) were diluted in 100 ml of PBS for nebulization.

Mice were placed in individual compartments of stainless steel wire cage exposure racks in two 135-liter chambers, one for LPS exposure and the other for filtered air. Animals were exposed 4 h/day, 5 days/week, for a total of 1 day, 5 days, or 8 wk. LPS solution was aerosolized with a constant-output atomizer (TSI model 3076, Minneapolis, MN) with all output directed to the exposure chamber. Filtered and dehumidified air was supplied to the nebulizer at 30 psi gauge pressure. The exposure chamber was vented at a flow rate of 35.0 l/min.

LPS assay. The airborne concentration of LPS was assayed by sampling 0.30–0.40 m³ of air drawn from the exposure chamber through 25-mm binder-free glass fiber filters (Gelman Sciences, Ann Arbor, MI) held within a 25-mm polypropylene inline air-sampling filter holder (Gelman). Filters were placed in pyrogen-free petri dishes with 2 ml of sterile PBS containing 0.05% Tween 20 (PBST; Sigma Chemical) and then placed on a rotating shaker at room temperature for 1 h. Aliquots of the wash solution were serially diluted in pyrogen-free water and tested for endotoxin by using a chromogenic Limulus amoebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, MD) according to the manufacturer’s instructions. The LPS concentration averaged 6,645 ± 204 EU/m³ (means ± SE) over the 8-wk exposure period. The LPS concentration for the single-day exposure was 4,469 ± 417 EU/m³ and averaged 6,167 ± 1,042 EU/m³ for the 5-day exposure.

Assessment of pulmonary function. Airway responsiveness to methacholine (MCH) challenge was assessed in unanesthetized mice by using whole body plethysmography. Individual mice were placed in plethysmograph chambers (Buxco Electronics, Troy, NY) ventilated by bias airflow at 1.0 ± 1.0·min⁻¹·chamber⁻¹. In each plethysmograph, a pressure signal was generated from the pressure difference of the main chamber containing the unrestrained mouse and a reference chamber, which canceled atmospheric disturbances. Signals were analyzed to derive whole body flow parameters (SFT3812; BioSystem XA version 2.0.2.48, Buxco), including respiratory rate, tidal volume, inspiratory and expiratory times (Ti, Te), peak inspiratory and expiratory flows (PIF, PEF), and relaxation time (RT). These parameters were used by the program software to calculate enhanced pause (Penh, unitless), which strongly correlates with lung resistance, and reflects changes in pulmonary resistance and airway smooth muscle constriction. Using the following expression: Penh = [(Te − RT)/RT]·PEF/PIF. Lung function was evaluated at baseline and after exposure to aerosolized MCH (0, 12.5, and 25 mg/ml for the 8-wk exposed group or 0, 5, 10, and 20 mg/ml for the 1- and 5-day groups). MCH was aerosolized for 1 min. Recording of breathing parameters began immediately after the end of MCH aerosolization and continued for 10 min. Average Penh values were determined over the first 3 min (early phase) of response to the MCH aerosol.

Whole lung lavage. Mice were euthanized by CO₂ inhalation, the chest was opened, the trachea was exposed, and lungs were lavaged through PE-90 tubing with 6.0 ml of sterile saline, 1 ml at a time, at a pressure of 20 cmH₂O. Return volume was recorded and was consistently greater than 4.5 ml. Processing of the lavage fluid has been described previously. 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 a small aliquot of this resuspension was used to count total lavaged cells per animal with a hemocytometer. One hundred microliters of the cell suspension were spun onto a slide with a cytocentrifuge (Shandon, Southern Sewickley, PA). Cells were stained with Hema-3 (Biochemical Sciences, Swedesboro, NJ) stain for differentials, air-dried, and covered with a coverslip with Cytoseal (Stephens Scientific, Kalama, MD).

Tissue preparation. After the lavage was completed, the lungs were perfused with saline through the pulmonary artery, and the whole right lung was removed and snap-frozen in liquid nitrogen and stored at −70°C for later use. Freshly prepared ice-cold 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) in 1× PBS (pH 7.4) was instilled through the tracheal cannula into the left lung at a constant pressure of 20 cmH₂O. The trachea was clamped, and the lung was fixed overnight at 4°C in 4% paraformaldehyde. This tissue was embedded in paraffin; 5- to 6-μm-thick sections were cut and placed on positively charged slides (Super Frost Plus, Fisher Scientific). Sections were stained with Masson’s trichrome (MEB; San Marcos, CA). Approximately 10 representative sections from each mouse lung were acquired with a Nikon Optiphot-2 microscope (Fryer, Huntley, IL) equipped with a Spot (Diagnostic Instruments, Sterling Heights, MI) digital camera and used for quantitative analysis.
**Morphometry.** Morphometry was performed by applying standard methods used before in our laboratory (7). Histological sections from both lavaged and un lavaged animals were evaluated in blinded fashion by experienced observers in this laboratory. There were no observable differences between the two groups in these evaluations. For each airway, external airway perimeter, basement membrane perimeter, and long and short airway diameters were measured on digital images captured at ×200 final magnification. The area of the submucosa was calculated by using these measurements. Airway profiles were divided into three relatively equal groups determined by airway short diameter: “small airways” (0–90 μm), “medium airways” (>90–129 μm), and “large airways” (>129 μm). For every airway measured, submucosal area was normalized to the length of the adjacent basement membrane. To minimize the error that might arise from tangential sectioning, any airway profiles showing a length-to-width ratio >2.5 were not used for analysis. The mean value of submucosal area standardized to length of basement membrane was calculated for each airway size for each study animal. These values were used to calculate means ± SE for each airway size for each study group.

**Immunohistochemistry.** BrdU immunohistochemistry was performed as described previously (1). Briefly, after deparaffinization and rehydration, sections were incubated in 2 N HCl for 20 min followed by two washes of 5 min each in PBST. After being washed, sections were incubated for 10 min in 0.4 mg/ml of pepsin (Sigma Chemical) in PBS at 37°C, followed by PBST washes. A rat monoclonal anti-BrdU antibody at dilution 1:100 was applied (Accurate Chemical and Scientific, Westbury, NY). The secondary antibody was biotinylated mouse anti-rat IgG (1:500; Jackson Immunoresearch Laboratories, West Grove, PA). After being washed again in PBST, sections were incubated in streptavidin-horseradish peroxidase (1:500). Immunoreactivity was visualized by using diaminobenzidine as the colorimetric substrate (Sigma Chemical).

**Quantitative analysis of cell proliferation.** One BrdU-stained histological section from each animal was prepared as described in Immunohistochemistry. For all airways in each BrdU-stained histological section from each animal, digital images were acquired and categorized with respect to size, as described in Morphometry. All airway epithelial and submucosal cells in each airway image were counted. Separate counts of BrdU-positive epithelial cells and BrdU-positive submucosal cells were also kept. The mean percent positive cells per airway for both of these anatomic compartments was calculated for each airway size for each study animal. These values were used to calculate means ± SE for each airway size for each study group.

**ELISA.** ELISA for IL-1β and TGF-β1 was performed on aliquots of lung lavage fluid from each animal in duplicate (IL-1β) or triplicate (TGF-β1) according to the manufacturer’s instructions. ELISA kits for IL-1β were purchased from R&D Systems (Minneapolis, MN). ELISA kits for TGF-β1 were purchased from Promega (Madison, WI). The lower limit of detection was 3.0 pg/ml for IL-1β and 15.6 pg/ml for TGF-β1.

**Statistical analyses.** All data are expressed as means ± SE. At each time point, the total and differential cell counts from lung lavage fluid, the airway responsiveness to inhaled MCh challenge, and morphometry were compared between endotoxin-sensitive (C3HeB/Fej) and -resistant (C3H/HeJ) mice. The differences between variables in each comparison were analyzed by ANOVA with Bonferroni’s post hoc comparisons for determination of significance between groups.
day. The lavage cellularity in C3HeB/FeJ mice exposed to endotoxin for both 1 day and 5 consecutive days consisted of >95% neutrophils (Fig. 2A). In contrast, C3H/HeJ mice had <5% neutrophils, which was not significantly different from air-exposed control mice. Even after 5 consecutive days of endotoxin exposure, there was no significant increase in the percentage of neutrophils present in the lavage fluid of exposed C3H/HeJ mice. Ninety-six hours after the end of the 8-wk exposure period, LPS-exposed C3HeB/FeJ mice had significantly elevated lavage cellularity compared with LPS-exposed C3H/HeJ mice and air-exposed mice from both strains (Fig. 2B). The majority of these cells were macrophages.

Morphometry. After the 8-wk endotoxin exposure period, C3HeB/FeJ mice exhibited significantly increased submucosal area in medium airways compared with endotoxin-exposed C3H/HeJ mice and control mice from both strains (Fig. 3). Additionally, in the small airways, endotoxin-exposed C3HeB/FeJ mice exhibited significantly enlarged submucosal area when compared with control C3HeB/FeJ mice but not compared with endotoxin-exposed C3H/HeJ mice. The submucosal area in large airways was not significantly different between exposed and unexposed mice in either strain.

Airway epithelial and submucosal cell proliferation. Because stereological measurements demonstrate significant submucosal thickening in small and medium airways in endotoxin-sensitive C3HeB/FeJ mice but not C3H/HeJ mice, we investigated whether cell proliferation resulting from elaboration of cytokines and growth factors contributes to submucosal thickening. Immediately after a single 4-h LPS inhalation, there was no difference between exposed C3HeB/FeJ and C3H/HeJ mice in the percentage of BrdU-positive epithelial or submucosal cells (Fig. 4, A and B). These values were similar to age-matched air-exposed control mice (data not presented) and previously reported results (1, 4). Immediately after 5 consecutive days of LPS inhalation, the percentage of epithelial and submucosal cells incorporating BrdU in small, medium,
and large airways in C3HeB/FeJ mice was significantly elevated compared with similarly exposed C3H/HeJ mice (Fig. 4, A and B). Ninety-six hours after the end of 2 mo of daily LPS exposure, the percent positive BrdU cells in the epithelium and submucosa of exposed C3HeB/FeJ mice was no longer significantly elevated (data not shown).

**TGF-β1 and IL-1β ELISA.** IL-1β levels in whole lung lavage fluid from exposed C3HeB/FeJ mice were significantly elevated compared with those from C3H/HeJ mice in which IL-1β was undetectable (Fig. 5). Immediately after 5 consecutive days of LPS inhalation challenge, the levels of IL-1β in C3HeB/FeJ mice had increased threefold compared with mice exposed for a single day, whereas IL-1β was still undetectable in C3H/HeJ mice. There was no significant difference between levels of total TGF-β1 in whole lung lavage fluid between the two mouse strains after a single endotoxin inhalation challenge (Fig. 6A). However, the levels of active TGF-β1 in C3HeB/FeJ mice were significantly elevated over levels in C3H/HeJ mice in which no active TGF-β1 was detectable (Fig. 6B). After 5 consecutive days of LPS inhalation challenge, levels of both active and total TGF-β1 were significantly elevated in C3HeB/FeJ mice compared with C3H/HeJ mice (Fig. 6, A and B). Ninety-six hours after the end of the 8-wk exposure protocol, there was no detectable TGF-β1 or IL-1β in whole lung lavage fluid from control or exposed mice of either strain (data not shown).

**DISCUSSION**

These studies demonstrate that long-term inhalation of LPS in endotoxin-responsive mice results in biological and physiological phenotypes characteristic of asthma and consistent with those observed after subchronic inhalation of grain dust (7). Here we demonstrate that structural changes in the lung associated with subchronic LPS exposure include a fibroproliferative process involving both epithelial and mesenchymal cells. We further demonstrate that IL-1β and TGF-β1 levels in lung lavage fluid from endotoxin-sensitive, but not endotoxin-resistant, mice continue to increase up to the intermediate 5-day time point in the exposure, potentially identifying a mechanism by which the fibroproliferative process is initiated and maintained. Our results suggest that cytokines and growth factors elaborated during the inflammatory response to inhaled endotoxin may prove to be fundamental to long-term changes in airway structure and function.

In this study, we show that subchronic inhalation of LPS enhances cell proliferation (as measured by BrdU incorporation) in epithelial and submucosal cells. In a recent study, it was demonstrated that pulmonary neutrophilic inflammation and injury resulting from inhalation of cigarette smoke or ozone in mice results in a significant increase in BrdU incorporation in the lung parenchyma and that this increase is dependent on IL-6 (32). Although not examined in this study, IL-6 is a proinflammatory cytokine known to be significantly increased over background levels in the lungs of mice challenged with inhaled grain dust (7) or intratracheally instilled LPS (31) and in humans challenged with inhaled LPS (13). Together, these studies suggest that IL-6 may play a critical role in initiating and sustaining the proliferative response to inhaled LPS and would further suggest a role for IL-6 in the repair process as a result of lung injury caused by inhaled LPS.

The role IL-1β may play in the persistent changes observed in grain dust or endotoxin-induced airway disease has not been clearly defined. Pulmonary edema fluid and, specifically, IL-1β from patients suffering from acute lung injury/acute respiratory distress syndrome was able to mediate epithelial cell spreading and proliferation in A549 cells in culture (5, 6). Moreover, the cell proliferation of A549 cells was demonstrated to be TGF-α dependent, suggesting that TGF-α may also participate in the process of LPS-induced airway remodeling (6). TGF-α is known to be a potent epithelial cell mitogen in culture (15) and has also been shown to have fibrogenic properties in TGF-α transgenic animals (10). In addition, an adenoviral vector

**Fig. 5.** Lung lavage fluid mean concentration of interleukin-1β protein immediately after 1 and 5 days of inhalation of LPS in C3H/HeJ and C3HeB/FeJ mice. Error bars are SE. *P < 0.05.

**Fig. 6.** Lung lavage fluid mean concentration of total (A) and active (B) transforming growth factor-β protein immediately after 1 and 5 days of inhalation of LPS in C3H/HeJ and C3HeB/FeJ mice. Error bars are SE. *P < 0.05.
delivering IL-1β to rat lung is sufficient to initiate and cause progression of airway remodeling, even after there is no longer any detectable IL-1β protein in whole lung lavage fluid from these animals (16). This process is mediated by increased expression of TGF-β1 that is induced consequent to IL-1β expression and that persists after IL-1β expression has returned to baseline levels (16). Together, these studies, in addition to our findings, suggest a mechanism by which long-term increases of inflammatory cytokines, such as IL-1β in mice, may cause initiation and progression of airway remodeling and that this mechanism may involve other fibrogenic growth factors, such as TGF-α and TGF-β1.

TGF-β1 is well known for its ability to induce mesenchymal cells to produce extracellular matrix components, such as fibronectin and collagen, and has been shown to be involved in fibrogenesis in a number of experimental model systems (1, 22–24). TGF-β1 has been demonstrated to induce formation of granulation tissue injected subcutaneously (24) and to increase expression of collagen in rat fibroblasts and human dermal fibroblasts in vitro (24). Furthermore, TGF-β1 is elevated in a mouse model of bleomycin-induced pulmonary fibrosis (25) and in mouse (1) and rat (22) models of asbestos-induced lung fibrosis. Finally, TGF-β1 and its signal transducer Smad2 have been shown to be upregulated in biopsies taken from human asthmatic subjects with evidence of airway remodeling (26), and the total number of neutrophils expressing TGF-β1 is higher in asthmatic vs. nonasthmatic patients (3). We demonstrate here that total and active TGF-β1 is present in significantly elevated levels in whole lung lavage fluid from endotoxin-exposed sensitive mice compared with levels in endotoxin-exposed resistant mice (Fig. 6). Together, these findings suggest that TGF-β1 may contribute to LPS-induced airway remodeling by virtue of its well-known fibrogenic properties.

In summary, this study has demonstrated that LPS-induced airway remodeling is a fibroproliferative process that may be dependent on IL-1β and TGF-β3 for its progression. It remains to be determined what factor or factors initiate and sustain the proliferative response observed in both the epithelium and the submucosa of affected airways. IL-6 appears to be a likely candidate, and further experiments are necessary to test this possibility. Differences in airway responsiveness, inflammation, and fibroproliferative responses between mouse strains sensitive and resistant to inhaled LPS suggest that specific molecules (IL-6, IL-1β, and TGF-β1) may be critical to the chronic airway changes that are characteristic of this environmental lung disease.

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DISCLOSURES

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