Cytokine gene expression after inhalation of corn dust

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Wohlford-Lenane, Christine L., Daniel C. Deetz, and David A. Schwartz. Cytokine gene expression after inhalation of corn dust. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L736–L743, 1999.—To characterize the time course and localization of the production of proinflammatory cytokines after inhalation of corn dust, we exposed mice (C3H/HeBFE) by inhalation challenge to sterile corn dust extract (CDE) and contrasted this response to inhalation of Escherichia coli 0111:B4 lipopolysaccharide (LPS) or pyrogen-free saline. After both CDE and LPS exposure, an increase in the concentration of bronchoalveolar lavage concentration of tumor necrosis factor (TNF)-α, interleukin (IL)-1α, and macrophage inflammatory protein (MIP)-2 resulted after inhalation of either CDE or LPS. Although the time courses of these cytokines were distinct, a similar pattern of release was observed after both CDE and LPS exposure. A single inhalation exposure of either CDE or LPS resulted in enhanced expression of mRNA for TNF-α, IL-1α, and MIP-2 that was evident and most pronounced within 1 h of the inhalation challenge. Although enhanced expression of mRNA for TNF-α was detectable 12 h after completion of the inhalation challenge, IL-1α and MIP-2 mRNA expression remained elevated through the 24-h time point. TNF-α, IL-1α, and MIP-2 expression was localized by in situ hybridization to inflammatory cells in the airways and alveoli from 1 to 24 h in both CDE- and LPS-exposed lungs. Interestingly, there was no convincing evidence that MIP-2 was substantially produced by airway epithelial cells. The pattern, timing, and location of expression of TNF-α, IL-1α, and MIP-2 mRNA after a single inhalation exposure of CDE in comparison with LPS is similar, supporting a common etiology and mechanism of inflammation in the lower respiratory tract. Moreover, our findings indicate that inhalation of corn dust or LPS results in an acute inflammatory process that is primarily mediated by inflammatory cells and appears to be self-limited.

endotoxin; lipopolysaccharide; kinetics; bronchoalveolar lavage

OCCUPATIONAL and environmental exposure to grain dust can cause a spectrum of clinical syndromes including asthma, acute and chronic changes in airway reactivity, bronchitis, and chronic obstructive lung disease (4). Among grain handlers, the prevalence of work shift changes in forced expiratory volume in 1 s (FEV1; ≥10% decline) varies between 3.9 and 11% (5, 10, 13). Exposure to grain dust causes seasonal decrements in airflow (23, 24, 28) and an accelerated longitudinal decline in FEV1 (6, 19, 29, 33, 41). Chronic bronchitis occurs in a high prevalence (23–37%) of grain handlers (14, 15, 19, 29). Moreover, acute changes in airflow across either the work shift or workweek are consistently associated with accelerated longitudinal declines in lung function (2, 6, 23, 33, 41). These observations indicate that grain dust-induced airway disease is a common occupational and environmental problem and that understanding the inflammatory response associated with acute inhalation may provide necessary information to prevent the chronic manifestations of grain dust-induced airway disease.

Although the pathogenesis of grain dust-induced airway disease is not entirely understood, the acute inflammatory response to inhaled grain dust involves recruitment of inflammatory cells, shedding of airway epithelial cells, and release of specific cytokines (8). After inhalation of grain dust, healthy, nonasthmatic, nonatopic study subjects without airway hyperreactivity develop significant declines in airflow (FEV1 decreases by 10–40%) that are associated with a neutrophilic alveolitis and an increase in the concentration of specific proinflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8) (8, 22). Inhalation studies in mice (11, 21, 37) have shown that after a single exposure to grain dust, neutrophils are rapidly recruited to the lung, and these cells produce and release proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokines (macrophage inflammatory protein (MIP)-1). However, the specific source of these mediators of inflammation are not known nor is the temporal pattern of production well defined.

These previous studies led us to hypothesize that acute injury to the airway epithelium and the associated airflow obstruction induced by grain dust is mediated by proinflammatory cytokines and chemokines that are produced and released from alveolar and airway macrophages, recruited polymorphonuclear neutrophils (PMNs), and airway epithelial cells. Furthermore, we speculate that the inflammatory response to grain dust is compartmentalized, with TNF-α and IL-1 (α and β) being primarily expressed by inflammatory cells (macrophages and PMNs), whereas MIP-2 (the murine homologue of IL-8 (40)) is produced by both inflammatory cells and the airway epithelium. The purpose of this study was to examine the kinetics and localization of proinflammatory cytokines (TNF-α and IL-1α) and a chemokine (MIP-2) that appear to be important in mediating the acute inflammatory response to inhaled corn dust in mice. This study represents an important step in determining whether these cytokines and chemokines play a role in grain dust-induced airway disease. Our results indicate that a single exposure to corn dust produces inflammation of the lower respiratory tract that is mediated by macrophages and PMNs, may persist for several days, and is very similar to the inflammatory response induced by similar doses of inhaled lipopolysaccharide (LPS). Additionally, although inflammatory cells in the lung actively produce TNF-α, IL-1α, and MIP-2, the airway epithelium does not appear to be involved in the production of these cytokines and chemokines.
METHODS

General experimental protocol. Endotoxin-sensitive (C3H/HeFb) mice were exposed for 4 h to either an aerosol of corn dust extract (CDE), LPS, or sterile saline in a 75-liter glass exposure chamber. After exposure, the mice were euthanized at selected time points and underwent whole lung lavage. The end of the exposure was designated as time 0. Lavage fluid was collected and analyzed for its cellularity and protein concentration. Whole lungs from animals not lavaged were dissected and either quick-frozen for RNA extraction or fixed in 4% paraformaldehyde followed by 20 and 30% sucrose and quick-frozen in liquid nitrogen for in situ hybridization.

Animals. Endotoxin-sensitive (C3H/HeFb) male mice (Jackson Laboratories, Bar Harbor, ME) were obtained at 6 wk of age and used within 2 wk of their arrival. The mice were maintained in an approved animal care facility. 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. Animal protocols were approved by the Institutional Animal Care and Use Committee.

CDE preparation. LPS was purchased as lyophilized purified Escherichia coli 0111:B4 (Sigma, St. Louis, MO). The corn dust used in these experiments was obtained from the air filtration systems at an eastern Iowa grain-handling facility. Sterile pyrogen-free saline was obtained from Baxter Medical Laboratories (Deerfield, IL).

CDE preparation. We prepared the extracts with standard methods (21) by adding 3.0 g of dust to 30.0 ml of Hank’s balanced salt solution, vortexing for 2 min, and shaking for 1 h at 4°C. Insoluble particles were removed from the suspension by centrifugation at 800 g for 20 min. CDE was passed through a sterile 0.22-µm-pore-size filter (Acrocap, Gelman Sciences, Ann Arbor, MI) to yield sterile CDE. The resulting filtrate was adjusted to pH 7.0, tested for sterility, and frozen at −70°C.

Endotoxin assay. The endotoxin concentrations of CDE solution, LPS solution, saline, and resulting aerosols were assayed with the chromogenic Limulus amoebocyte lysate assay (QCL-1000, BioWhittaker, Walkersville, MD) with sterile pyrogen-free labware and a temperature-controlled microplate block and microplate reader (405 nm). The extracts and LPS solutions were serially diluted in pyrogen-free water (pfw) and assayed. The airborne concentration of endotoxin was measured by sampling 0.30 m³ of air drawn from the exposure chamber through 47-mm binder-free glass microfiber filters (EPM-2000, Whatman, Maidstone, UK) held within a 47-mm stainless steel-in line air-sampling filter holder (Gelman Sciences). Air-sampling filters were extracted with 10 ml of pfw at room temperature with gentle shaking for 1 h. The extracts were then serially diluted with pfw and assayed for endotoxin. Four to six air samples were assayed for each exposure. All standard curves achieved a linear regression coefficient exceeding r = 0.999. Our laboratory routinely runs spiked samples and filter blanks and participates in interlaboratory validation studies. The concentration of endotoxin was 0 µg/ml in the saline aerosol, 8.3 µg/ml for the CDE aerosol, and 7.5 µg/ml for the LPS aerosol.

Exposure and monitoring apparatus. CDE and LPS aerosols were generated and directed into a glass 75-liter exposure chamber by use of a PITT no. 1 nebulizer. Chloroform was added, the total RNA was precipitated, and dehydrated with ethanol. Slides were fixed for 10 min in 4% paraformaldehyde, rinsed with 2 × saline-sodium citrate (SSC), and digested with 1 µg/ml proteinase K. To acetylate amino groups in the tissue and reduce the electrostatic nonspecific binding of the probe, we incubated the slides in triethanolamine buffer containing acetic anhydride (0.25%). Sections were rinsed with 2 × SSC and dehydrated with ethanol. Slides were hybridized overnight at 50°C in a hybridization buffer containing 12.0 × 10³ counts/min-1·µl labeled probe-1 (42). After hybridization, the slides were rinsed in 4 × SSC containing 10 mM 1,4-dithiothreitol at 50°C until the coverslips fell off. The slides were then rinsed in an RNase solution, dehydrated through an ethanol series, and air-dried. Autoradiography was performed with NTB-2 liquid emulsion (Eastman Kodak, Rochester, MN) and exposed to X-ray film at −70°C. Densitometry was performed to control for the concentration of RNA in each lane with Sigma gel (Jandel Scientific Software, San Rafael, CA).

In situ hybridization. Eight-micrometer-thick paraformaldehyde-fixed frozen sections were cut at −20°C and mounted onto Superfrost Plus glass slides. Slides were fixed for 10 min in 4% paraformaldehyde, rinsed in 2 × saline-sodium citrate (SSC), and digested with 1 µg/ml proteinase K. To acetylate amino groups in the tissue and reduce the electrostatic nonspecific binding of the probe, we incubated the slides in triethanolamine buffer containing acetic anhydride (0.25%). Sections were rinsed with 2 × SSC and dehydrated with ethanol. Slides were hybridized overnight at 50°C in a hybridization buffer containing 12.0 × 10³ counts/min-1·µl labeled probe-1 (42). After hybridization, the slides were rinsed in 4 × SSC containing 10 mM 1,4-dithiothreitol at 50°C until the coverslips fell off. The slides were then rinsed in an RNase solution, dehydrated through an ethanol series, and air-dried. Autoradiography was performed with NTB-2 liquid emulsion (Eastman Kodak, Rochester, MN).
ter, NY) diluted 1:1 with distilled water at 42°C. Slides were coated with emulsion, allowed to dry, packed in a lighttight slide box containing desiccant, and stored at 4°C. Slides were developed in D19 developer for 3 min, rinsed in distilled water for 30 s, and fixed for 3 min. Slides were counterstained in hematoxylin and dehydrated through an ethanol series. The following cDNA clones were used to generate 35S-labeled sense and antisense cRNA probes for the in situ studies: murine (mu) TNF-α (pMuTNF, American Type Culture Collection, Mannasas, VA), muIL-1α (pMuIL1α cDNA, American Type Culture Collection), and muMIP-2 (a kind gift from Dr. Barbara Sherry, Laboratory of Medical Biochemistry, Rockefeller University, New York, NY). All cDNA clones were cloned into a pBluescript II SK vector (Stratagene, La Jolla, CA).

Statistical analysis. Comparisons were performed to investigate the kinetics of the inflammatory response to inhaled CDE and LPS. At each time point, for each response, the concentrations of lavage cells and cytokines were compared with those of saline-exposed mice. In addition, the relative effect of CDE and LPS was evaluated by directly comparing the inflammatory response of CDE and LPS at each time point after the inhalation challenge. The Mann-Whitney U-test was used to assess significance of these comparisons (35).

RESULTS

Similar cellular inflammatory changes in the lower respiratory tract were observed after exposure to CDE and LPS (Fig. 1). Moreover, the kinetic profile of cellular changes in the lavage fluid was remarkably similar after exposure to either CDE or LPS. A marked increase in the concentration of PMNs was noted in the lavage fluid after exposure to either of these agents. The concentration of PMNs peaked at 1 h after exposure to CDE and 4 h after inhalation of LPS. Elevations in lavage concentration of PMNs persisted for 48 h. A small but significant elevation in lavage macrophage concentration was present 48 h after exposure to CDE but not to LPS. In addition, a small but significant increase was observed in the concentration of lymphocytes after inhalation of either CDE (4–48 h) or LPS (24–48 h). There were no significant changes in the concentration of eosinophils in the lavage fluid at any time point (data not shown). No significant differences were observed in the cellular inflammatory responses between CDE and LPS at any of the time points postexposure. At 96 h after inhalation of either CDE or LPS, the concentrations of PMNs, macrophages, and lymphocytes were similar to baseline or preexposure levels.

Inhalation of CDE and LPS resulted in significant increases in the concentration of proinflammatory cytokines in the lavage fluid of mice (Fig. 2). Again, the kinetic profile of cytokine inflammatory changes in the lavage fluid was similar after exposure to either CDE or LPS. Concentrations of inflammatory mediators TNF-α, IL-1α, and MIP-2 were significantly increased in the initial postexposure lavage fluid for both CDE and LPS. Lavage fluid TNF-α concentrations peaked at 1 h for CDE inhalation and 4 h for LPS inhalation and remained significantly elevated for 48 h after inhalation.
of either CDE or LPS. Lavage IL-1α and MIP-2 concentrations peaked at 1 h after inhalation of either CDE or LPS. IL-1α levels returned to baseline at 96 h after the inhalation challenge. For CDE inhalation, MIP-2 levels returned to baseline at 48 h, and for LPS, MIP-2 levels returned to baseline at 24 h.

Similar patterns of mRNA cytokine expression for TNF-α, IL-1α, and MIP-2 were observed in the lungs after inhalation of CDE and LPS at each of the time points (Fig. 3). TNF-α mRNA was expressed in CDE- and LPS-exposed mouse lungs at 1 h (the earliest time point) and returned to near baseline at 24 h. IL-1α and MIP-2 mRNA expression peaked at 1 h and continued to be expressed through 24 h after exposure to either CDE or LPS. No cytokine mRNA expression was observed in the lungs of saline-exposed mice at any time point postinhalation. Comparing densitometry measures and controlling for the total RNA in each sample, we observed no substantial difference in the induction or kinetics of mRNA for specific cytokines after inhalation of CDE or LPS (densitometry values not presented).

TNF-α, IL-1α, and MIP-2 mRNAs were detectable in CDE- and LPS-exposed mouse lungs with the use of 35S-labeled antisense cRNA probes for in situ hybridization (Figs. 4–6, respectively). TNF-α and IL-1α mRNAs were present in inflammatory cells scattered throughout the airways and alveoli from 1 through 12 h. This was observed for samples obtained after inhalation of either CDE or LPS but was not observed after inhalation of saline. In situ hybridization of lavage cytospins confirmed expression of TNF-α and IL-1α mRNAs by macrophages and PMNs. Occasional hybridization was seen at 24 h for TNF-α and IL-1α but at a much lower level; the persistence of mRNA for TNF-α and IL-1α as assessed by in situ hybridization (in comparison with the results from the RNase protection assay) is most likely due to the increased sensitivity of in situ hybridization. MIP-2 was present in inflammatory cells throughout the airway from 1 through 24 h in lung tissue in both CDE- and LPS-exposed samples. Interestingly, the airway epithelium does not convincingly express mRNA for MIP-2 after inhalation of either CDE or LPS (Fig. 7). When tissue from CDE-, LPS-, and saline-exposed lungs was hybridized with 35S-labeled TNF-α, IL-1α, or MIP-2 sense cRNA probes, no message was detected at any time point (Figs. 4–6). Also, there

![Fig. 2. Concentration of lavage cytokines tumor necrosis factor (TNF)-α (A), interleukin (IL)-1α (B), or macrophage inflammatory protein (MIP)-2 (C) in mice after a single inhalation challenge with CDE, LPS, or saline. Values are means ± SE. *P < 0.05 for CDE or LPS compared with saline exposure at each time point.](image)
Fig. 4. In situ hybridization of mouse lungs with TNF-α 4 h after inhalation challenge with saline (A–C), LPS (D–F), or CDE (G–I). Hybridizations are antisense, bright field (A, D, and G); antisense, dark field (B, E, and H); and sense, dark field (C, F, and I). TNF-α is produced by inflammatory cells (macrophages and neutrophils) scattered throughout airway (aw) and alveoli. Arrows, representative areas of hybridization. Original magnification, ×200.

Fig. 5. In situ hybridization of mouse lungs with IL-1α 4 h after inhalation challenge with saline (A–C), LPS (D–F), or CDE (G–I). Hybridizations are antisense, bright field (A, D, and G); antisense, dark field (B, E, and H); and sense, dark field (C, F, and I). IL-1α is produced by inflammatory cells (macrophages and neutrophils) scattered throughout aw and alveoli. Arrows, representative areas of hybridization. Original magnification, ×200.
was no expression of mRNA for TNF-α, IL-1α, or MIP-2 in lungs obtained from mice exposed to saline.

DISCUSSION

The results from our study indicate that inhaled corn dust causes an inflammatory response in the lower respiratory tract of mice that is similar to that with inhaled LPS. These inflammatory responses persist for several days after the inhalation challenge, are largely mediated by airway inflammatory cells, and are ultimately self-limited. Although airway inflammatory cells produce and release TNF-α, IL-1α, or MIP-2 into the air spaces, the airway epithelium does not appear to be involved in the production or release of any of these proinflammatory proteins.

The results presented in this study demonstrate that the kinetics and pattern of cell recruitment and cytokine release and/or expression are remarkably similar in mice after inhalation of corn dust or LPS. These findings further support a growing body of literature that indicates that endotoxin is one of the primary agents in grain dust that causes airway inflammation and airflow obstruction. Previously, our laboratory (38) has shown that the concentration of endotoxin in the bioaerosol appears to be the most important occupational exposure associated with the development of airway disease in grain handlers. Physiologically, inhaled endotoxin (3, 17, 30, 31, 36) or grain dust (8, 9, 12) can cause airflow obstruction in naive or previously unexposed subjects. Our laboratory's previous exposure-response studies have shown that inhaled grain dust and endotoxin produce similar physiological and biological effects (8, 22), the concentration of endotoxin appears to play an important role in the acute biological response to grain dust (21, 22, 37), a competitive antagonist for LPS (Rhodobacter sphaeroides diphosphoryl lipid A) reduces the inflammatory response to inhaled grain dust (20), and genetic or acquired hyporesponsiveness to endotoxin substantially reduces the biological response to grain dust (37). In aggregate, these findings, in conjunction with those presented...
macrophages are the primary source of TNF-α. Xing and colleagues who observed that PMNs and macrophages are essential to the release of these cytokines. This hypothesis is supported by findings by Schraufnagel and colleagues who observed that PMNs and macrophages are the primary source of TNF-α (44) and MIP-2 (43) in a rat model of acute inflammation induced by intratracheal instillation of LPS. Furthermore, our results indicate that the airway epithelium is not involved in the production of MIP-2. Importantly, epithelial cells (in vitro) respond poorly to LPS and appear to require a specific host-derived signal (TNF-α or IL-1) for induction of IL-8 (1, 27, 32, 34, 39, 40), the human chemokine equivalent of MIP-2 (21). The inability to respond to LPS has been demonstrated in vitro in A549 cells (39) and bronchial epithelial cells (27, 32); however, if these cells are exposed directly to either TNF-α or IL-1, they are able to produce and release IL-8. In a baboon model of sepsis, pretreatment with anti-TNF-α antibody significantly reduced the circulating concentration of IL-8 (42), suggesting that TNF-α and/or IL-1 is needed to stimulate other cells to release IL-8 and promote neutrophil chemotaxis. However, other causes of lung inflammation, such as IL-1β (45) and α-quartz (16), have been shown to directly induce airway epithelia to upregulate the expression of MIP-2 mRNA. Our findings suggest that inhaled organic dust initiates a complex interaction between macrophages and other inflammatory cells (primarily PMNs) mediated by specific proinflammatory cytokines released by these cells. Because MIP-2 is not produced by the airway epithelium, other factors such as adhesion proteins must function to localize the inflammatory cells to the airway. Alternatively, the airway epithelium may produce MIP-2 at a much earlier time point. Because our inhalation protocol takes 4 h and the initial specimens are obtained 1 h after the exposure ends, it is conceivable that MIP-2 (or any of the other cytokines) was produced by the airway epithelium before the sample period.

A key, unanswered question is the relationship between the acute reversible inflammatory and physiological response to inhaled grain dust and the development of chronic airway disease (4). Our model of grain dust-induced airway disease represents an excellent opportunity to study this relationship for the following reasons. First, we have developed a reliable and valid model of the acute physiological and biological response to inhaled grain dust in humans (8, 11, 22) and mice (11, 20, 21, 37). Second, our laboratory (11) has shown that the acute physiological and inflammatory response to inhaled grain dust is self-limited, with airflow obstruction persisting for 48 h, and the inflammatory response, most prominent within hours of exposure, ultimately resolves. These findings indicate that a single inhalation challenge of grain dust results in airflow obstruction and lower respiratory tract inflammation that may last for several days but is self-limited, completely resolving 1 wk after challenge. Third, our laboratory (11) demonstrated similar inflammatory responses in mice and humans after inhalation of grain dust, suggesting that the mouse model provides an opportunity to study the pathogenesis of grain dust-induced airway disease in humans. Fourth, as reviewed above, endotoxin appears to be one of the primary agents in grain dust, causing airflow obstruction and airway inflammation. On the basis of this finding, one could develop specific interventions directed at either endotoxin or the cytokines involved in the inflammatory response to endotoxin to downregulate the inflammatory response to inhaled grain dust. Fifth, chronic exposure to grain dust causes accelerated longitudinal declines in FVC (6, 19, 29, 41), is associated with a high prevalence of chronic bronchitis handlers (14, 15, 19, 29), and causes irreversible and progressive airway disease (26). Finally, because acute changes in airflow across either the work shift or week are consistently associated with accelerated longitudinal declines in lung function (6, 23, 41), modifying the acute response to inhaled grain dust is likely to alter the risk of developing chronic grain dust-induced airway disease.

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