

Grain Dust and Endotoxin Inhalation Challenges Produce Similar Inflammatory Responses in Normal Subjects*

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Study objective: **To compare the physiologic and inflammatory response following inhalation of corn dust extract (CDE) and lipopolysaccharide (LPS) solutions in normal subjects.**
Design: **Randomized, double-blind crossover design.**
Participants: **Fourteen healthy, nonatopic, nonasthmatic, never-smoking volunteers.**
Interventions: **On separate visits, subjects underwent a series of four inhalation challenges to LPS or CDE, each containing either a high (6 μg/mL) or low (0.9 μg/mL) endotoxin concentration, and administered at equal volumes.**
Results: **Chest tightness, cough, dyspnea, and sputum production were experienced following both LPS and CDE exposures and with similar frequency at both high and low endotoxin concentrations. LPS and CDE inhalations caused acute declines in FEV_1, and the changes in FEV_1 from baseline following exposure to both inhalants were not significantly different at both high and low endotoxin concentrations. Following exposure to the high-endotoxin LPS and CDE, no consistent differences in total cell and cytokine (tumor necrosis factor-α [TNF-α], interleukin-1β [IL-1β], IL-6, IL-8) concentrations were seen between exposures, although the neutrophil concentration was greater following the LPS exposure ($p=0.01$). BAL cellularity and cytokine concentrations following the low-endotoxin LPS and CDE exposure revealed no differences, except for IL-1β, which was greater following LPS exposure ($p=0.05$). The high-endotoxin LPS and CDE exposures resulted in greater increases in BAL neutrophils and cytokines in comparison to its respective low-endotoxin exposure.**
Conclusions: **At equivalent exposure levels of endotoxin, LPS and CDE result in similar symptoms, changes in airflow, and increases in BAL inflammatory cells and mediators. Moreover, the physiologic and inflammatory response to LPS and CDE appears to be related to the exposure level of endotoxin.** ***(CHEST 1996; 110:263-70)***

Key words: airflow obstruction; airway inflammation; endotoxin; grain dust; inhalation

Abbreviations: CDE=corn dust extract; cDNA=complementary DNA; IL=interleukin; LPS=lipopolysaccharide; mRNA=messenger RNA; PCR=polymerase chain reaction; TNF=tumor necrosis factor

Previous epidemiologic investigations have demonstrated the adverse effects from long-term inhalation of grain dust on respiratory symptoms and lung function.[1-6] Specifically, there is a high prevalence of chronic bronchitis (23 to 33%)[2,3] and increased bronchial reactivity.[7] Long-term grain dust exposure causes accelerated longitudinal declines in lung function, and a dose-response relationship exists between long-term grain dust exposure and the development of both respiratory symptoms and declines in lung function.[8,9]

Given the chronic respiratory effects following grain dust inhalation, investigation into the potential mechanisms by which grain dust stimulates airway inflammation represents a relevant area of research. Prior studies have demonstrated that volunteer subjects not previously exposed to grain dust develop acute declines in airflow following a single inhalational exposure to

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Table 1—*Frequency of Symptoms Reported Following Inhalation Exposure

Symptoms	High Endotoxin Exposure (n=14)			Low Endotoxin Exposure (n=12)		
	LPS	CDE	p Value†	LPS	CDE	p Value†
Chest tightness	12 (86)	9 (64)	NS	7 (58)	7 (58)	NS
Cough	8 (57)	7 (50)	NS	5 (42)	5 (42)	NS
Phlegm	4 (29)	5 (36)	NS	4 (33)	6 (50)	NS
Dyspnea	3 (21)	2 (14)	NS	1 (8)	1 (8)	NS

*Values represent actual number of subjects reporting symptoms; the percentage of all subjects reporting symptoms is shown in parentheses.
†NS=p value>0.05.

extracts of grain dust.[10] Thus, it appears that grain dust inhalation causes airway inflammation primarily through nonallergic mechanisms.

One agent that may be responsible for the inflammatory effects of grain dust is endotoxin. Endotoxin has been measured consistently at significant levels in samples of grain dust taken from grain elevators.[11] Endotoxin has been shown to cause decreases in forced expiratory flow rates and cause airway inflammation.[12,13]

To better understand the relationship between endotoxin and grain dust-induced airway disease, healthy volunteers underwent inhalation challenges to corn dust extract (CDE) and lipopolysaccharide (LPS), both containing equal endotoxin activity, to determine whether differences exist in the characteristics of the physiologic or inflammatory response to these agents. We hypothesize that if endotoxin is the principal substance responsible for the development of lung inflammation and airflow obstruction in grain dust-induced airway disease, the characteristics of the physiologic or inflammatory response in subjects inhaling CDE will be very similar to an LPS solution containing equal endotoxin activity. Moreover, these physiologic and inflammatory changes following inhalation challenge will be significantly influenced by altering the concentration of endotoxin in the CDE.

Materials and Methods

We used a double-blind, randomized, crossover design to compare the physiologic and inflammatory response to inhalation of CDE to LPS solution containing equivalent concentrations of endotoxin activity.

Study Subjects

Healthy, never-smoking subjects without any history of cardiopulmonary disease or history of occupational exposure to grain dust were recruited. The study protocol used was approved by the human subjects committee at this institution. To qualify, study subjects were required to have normal results of a physical examination, 12-lead ECG, chest radiograph, pulmonary function tests (spirometry, lung volumes, diffusing capacity, and arterial blood gases), and normal bronchial reactivity by histamine challenge.[14,15] Subjects underwent skin testing (Greer DermaPik System; Greer Laboratories; Lenoir, NC) and were excluded if a positive reaction to any allergen was obtained.

Protocol

Study subjects underwent a series of four inhalation challenges, with exposures being separated by at least 3 weeks. In the first 2 exposures, subjects were randomized to inhale either CDE or LPS, each containing an equivalent concentration of endotoxin (6.0 μg/mL). In the third and fourth exposures, subjects were randomized to inhale CDE or LPS solutions containing a lower endotoxin concentration (0.9 μg/mL). Spirometry was measured prior to and following exposure to each inhalant. Bronchoscopy was performed 4 h following completion of the inhalation challenge.

Preparation of Inhaled Solutions

CDE was prepared by mixing settled corn dust in sterile, pyrogen-free Hanks' balanced salt solution (0.1% solution) by methods we have described previously.[16] The measured endotoxin concentration in this CDE was 0.9 μg/mL by the end point chromogenic Limulus amebocyte lysate assay (QCL-1000; Whittaker Bioproducts; Walkersville, Md). This was designated as the low-endotoxin CDE. To increase the concentration of endotoxin in the CDE solution without altering the other components in the CDE, a high-endotoxin CDE was prepared by adding *Escherichia coli* (serotype 0111:B4) LPS (Sigma Chemical Co; St. Louis) to a separate aliquot of the low-endotoxin CDE solution to obtain a final endotoxin concentration of 6.0 μg/mL. This was designated as the high-endotoxin CDE solution. At this higher endotoxin concentration, it was possible for subjects to be exposed over a 60-min exposure period to an endotoxin concentration between 30 and 60 μg, which is within the expected range of endotoxin exposure measured in grain workers over an 8-h workshift.[17,18] LPS solutions designated as high (6.0 μg/mL) and low (0.9 μg/mL) LPS were prepared separately by resuspending lyophilized *E coli* LPS in sterile Hanks' balanced salt solution. All solutions used for inhalation were derived from a stock solution, which underwent sterility testing (bacteria and fungi) and endotoxin assay prior to separation into individual aliquots. These aliquots were stored at −70°C until being used.

Inhalational Challenge

The solutions were administered via a nebulizer (DeVilbiss 646) and dosimeter (DeVilbiss; DeVilbiss Health Care Inc; Somerset, Pa), operated at 20 psi air pressure. For each exposure, subjects were administered 0.1 mL of inhalant per kilogram of body weight, given over 60 min.

Pulmonary Function Testing

The pulmonary function tests consisted of serial spirometry using a spirometer (Spirotech S-600; Graseby Anderson; Atlanta). These maneuvers were performed using standard protocols and the American Thoracic Society guidelines.[19] Spirometry was performed preexposure and at the following time points postexposure: 10, 20, and 30 min, and 1, 2, 3, 4, and 24 h.

Bronchoscopy

Bronchoscopy was performed in accordance with the standards established by the American Thoracic Society for bronchoscopy in asthmatics.[20] A fiberoptic bronchoscope (Olympus P-10; Olympus; Lombard, Ill) was introduced into the chosen lung segment for lavage and wedged. Twenty milliliters of sterile, 0.9% saline solution (37°C) was injected through the bronchoscope, repeating 5 more times for a total lavage volume of 120 mL. The return of the first 20-mL aliquot was separated from the remaining lavage fluid. Lung segments chosen for BAL alternated between a subsegment of the right middle lobe following the first and third exposures and a subsegment of the lingula following the second and fourth exposures. Endobronchial brushings of a mainstem bronchus were collected following BAL.

Processing of Specimens

BAL samples were processed according to methods described previously.[10] BAL cell differential counts were quantified from stained cytospin preparations, counting 200 cells; 1×10^6 cells were pelleted for subsequent RNA extraction. Tumor necrosis factor (TNF-α) bioactivity in BAL supernatant fluid and in serum was measured with a standard cytotoxicity assay using TNF-sensitive L929 cells.[21] Interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interleukin-8 (IL-8) were measured in the BAL supernatant fluid and in serum using commercially available enzyme immunoassays (R&D Systems; Minneapolis).

Semiquantitative Polymerase Chain Reaction (PCR) Determination of Relative Cytokine mRNA Levels

Methods used to determine the relative cytokine messenger RNA (mRNA) for TNF-α and IL-8 in the BAL and epithelial cells were followed as previously described.[10,22] The β-actin primer pair corresponded to the bp 835-856 (sense) and bp 1015-1035 (antisense) of the β-actin complementary DNA (cDNA) sequence,[23] TNF-α primer pair was synthesized according to sequences described by Wang et al,[24] and the IL-8 primer pair was made using bp 147-174 (sense) and bp 324-266 (antisense) of the IL-8 cDNA sequence.[25] A standard curve (1:5 dilutions) of cDNA derived from alveolar macrophages stimulated with endotoxin *in vitro* (1 μg/mL for 6 h) was amplified in parallel with the unknown cDNAs. One mRNA unit corresponded to RT-PCR product derived from 1 ng macrophage RNA. cDNA was amplified in a 96-well thermocycler (MJ Research; Watertown, Mass), 94°C-1 min, 56°C-2 min, 72°C-2 min, for 30 to 35 cycles. The PCR products were electrophoresed into 2% agarose gels containing ethidium bromide and photographed. The negatives were scanned using an image scanner and software package (Hewlett Packard Scan Jet II and Desk Scan II; Boise, Idaho), and optical density measurements were performed. Sample mRNA units were derived from the macrophage standard curves run for the same number of cycles.

Statistics

Two primary comparisons were made: (1) comparison of LPS to CDE at both high and low concentrations of endotoxin for spirometric changes and biological measures of inflammation (BAL cellularity and BAL and serum cytokines); and (2) comparison of the spirometric and biological response between LPS and CDE solutions containing high and low concentrations of endotoxin. Analysis of data was performed using paired, nonparametric statistics (Wilcoxon signed rank test).[26] A χ^2 analysis was performed in comparing symptom frequencies between inhalational challenges.[26]

Results

Fourteen subjects, 9 men and 5 women, participated in the study, with subjects' ages ranging from 20 to 48 years (mean age, 30 years). All subjects completed a paired (CDE-LPS) exposure at the high endotoxin concentration (6 μg/mL), and 12 subjects completed a second paired (CDE-LPS) exposure at the low endotoxin concentration (0.9 μg/mL). Two subjects did not wish further participation because of the time commitment.

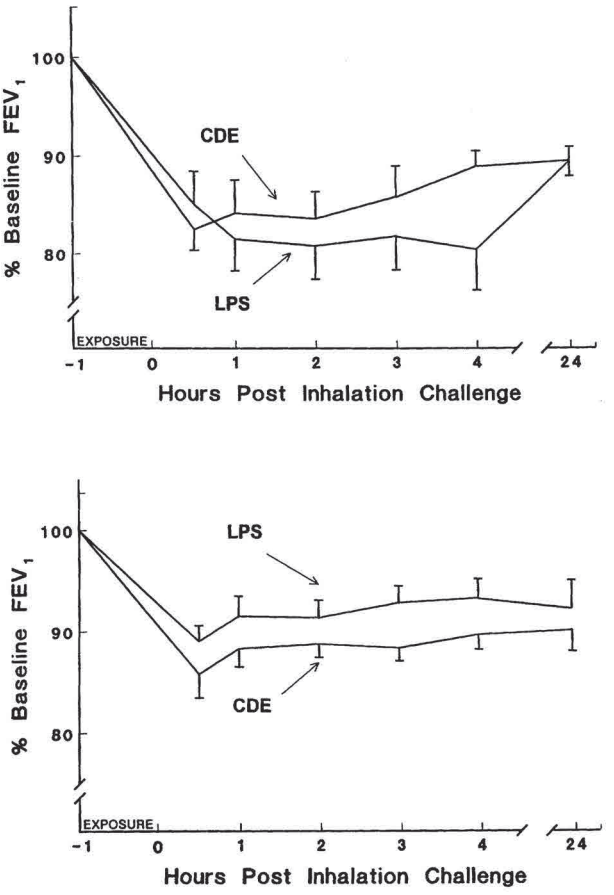


FIGURE 1. The mean (±SEM) percent baseline FEV_1 is depicted over time in subjects following inhalation challenges with the high endotoxin (6 μg/mL) *(top)* and low endotoxin (0.9 μg/mL) *(bottom)* LPS and CDE solutions.

Following inhalation exposure to both the high and low endotoxin CDE and LPS solutions, the most common symptoms reported were chest tightness, cough, sputum production, and dyspnea (Table 1). Chest tightness was the most frequently experienced symptom, occurring in 86% and 64% of subjects exposed to high-endotoxin LPS and CDE solutions, respectively, and at least half of these subjects experienced coughing. In subjects inhaling the low endotoxin LPS and CDE solutions, chest tightness (58%) and cough (42%) occurred with equal frequency. Overall, there was no statistical differences in the frequency in which each symptom was reported when comparing LPS with CDE at equivalent endotoxin concentrations as well as when comparing the high endotoxin LPS or CDE exposure with its low endotoxin counterpart.

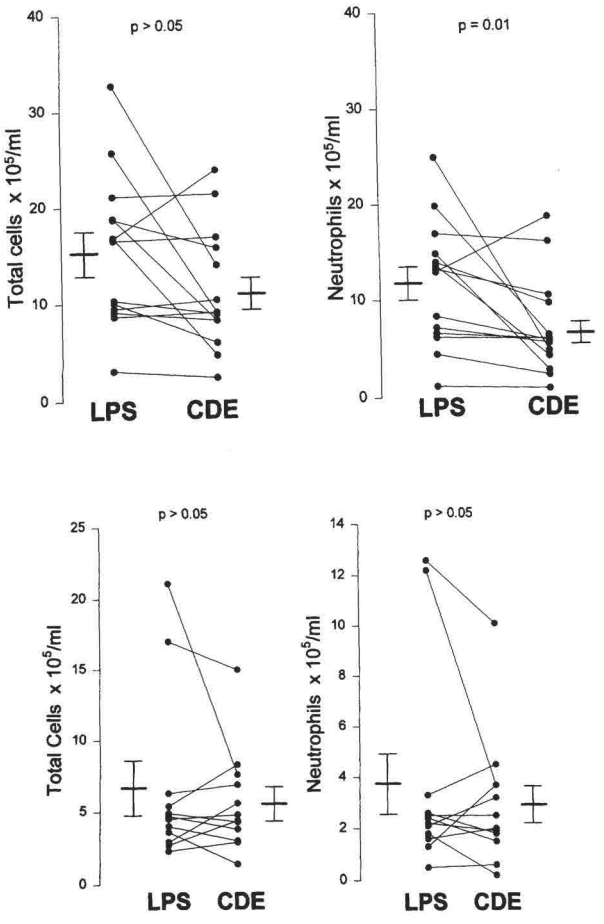


FIGURE 2. BAL total cell and neutrophil concentrations are shown for subjects following inhalation challenge to the high endotoxin *(top)* and low endotoxin *(bottom)* LPS and CDE solutions. The group mean (±SEM) is represented for each exposure.

Inhalation of LPS and CDE at high endotoxin concentrations resulted in a significant decline in FEV_1 from baseline (Fig 1, *top*), beginning as early as 10 min after completion of the exposure to both inhalants (data at 10 and 20 min not shown). As a group, the mean percentage declines in FEV_1 from baseline in subjects exposed to LPS were 17 to 19% and for CDE 10 to 16% during the first 4 h following inhalation exposure. No differences ($p>0.05$) in the percent declines in FEV_1 from baseline were found between LPS and CDE at time points 1, 2, 3, 4, and 24 h. Subjects exposed to low endotoxin containing LPS and CDE resulted in significant declines in FEV_1 from baseline (Fig 1, *bottom*). As a group, the mean percent declines from baseline within the first 4 h following inhalation exposure ranged from 7 to 9% for LPS and 10 to 14% for CDE. The 12 subjects exposed to both the high and low endotoxin LPS solutions demonstrated significantly greater percent declines in FEV_1 from baseline at 1, 2, and 3 h time points following exposure to the high-endotoxin LPS solution in comparison to the low endotoxin LPS solution ($p<0.03$ for each time point). In contrast, there was no significant difference in the declines in FEV_1 at any of the time points examined between the high and low endotoxin CDE exposure.

Inhalation of the high-endotoxin LPS and CDE solutions resulted in an acute inflammatory response in the lung 4 h following exposure to these agents. Comparison of BAL cellularity (Fig 2, *top*) revealed no significant difference in the total concentration of cells. However, greater concentrations of neutrophils were observed following exposure to LPS in comparison to CDE ($p=0.01$). Subjects exposed to the low endotoxin LPS and CDE inhalants also developed increases in the mean concentration of total cells and neutrophils, although as a group, these concentrations were approximately half in comparison to the high endotoxin LPS and CDE exposures. When comparing an individual subject's response to low-endotoxin LPS and CDE exposures, there was no statistical difference in the total cell or neutrophil concentrations between exposures (Fig 2, *bottom*) ($p>0.5$). In subjects completing all four exposures, individual BAL total cell and neutrophil concentrations were significantly greater ($p<0.02$) in response to inhalation of the high endotoxin LPS or CDE solution in comparison to its respective low endotoxin-containing counterpart.

TNF-α, IL-1β, IL-6, and IL-8 concentrations were all increased in the BAL fluid 4 h following inhalation of the high and low endotoxin LPS and CDE solutions. However, when comparing an individual's response to the high endotoxin LPS and CDE solutions, there was no difference in the concentrations of TNF-α, IL-1β, IL-6, and IL-8 measured in BAL fluid (Fig 3, *top*). Similarly, comparison of an individual's response to the low endotoxin LPS and CDE solutions revealed no difference in the concentrations of TNFα, IL-6, and IL-8 (Fig 3, *bottom*). However, BAL concentrations of IL-1β were found to be statistically greater following LPS inhalation in comparison to CDE ($p=0.05$). In subjects completing all four exposures, the high-endotoxin LPS exposure caused significantly greater ($p<0.01$) concentrations of TNF-α and IL-8 in the BAL fluid in comparison to the low endotoxin LPS exposure, and the high endotoxin CDE exposure resulted in greater ($p<0.01$) concentrations of TNF-α, IL-1β, and IL-8 in comparison to the low endotoxin CDE exposure.

Of the cytokines detected in BAL fluid following exposure to LPS and CDE, only IL-6 was detected in the serum 4 h following exposure to LPS and CDE at high and low endotoxin concentrations. However, no significant differences in serum IL-6 concentrations were seen when comparing an individual subject's response to LPS and CDE at either high or low endotoxin concentrations, respectively (Fig 4). As a group, mean serum concentrations were much greater

following the high endotoxin exposures than in the low endotoxin exposures. Serum IL-6 level was significantly greater in subjects following exposure to the high endotoxin-containing inhalants (LPS or CDE) in comparison to levels measured in the same subjects following exposure to its respective low endotoxin-containing counterpart.

Semiquantitative RT-PCR of BAL and epithelial cells demonstrated the expression of TNF-α mRNA in BAL cells and IL-8 mRNA in airway epithelial cells following exposure to both LPS and CDE (Fig 5). Comparison of relative amounts of mRNA expressed revealed no significant differences in TNF-α mRNA expression in BAL cells or IL-8 mRNA expression in airway epithelial cells between LPS and CDE exposures for both high and low endotoxin exposures (Fig 6). Although mRNA concentrations of TNF-α in BAL cells and IL-8 expression in airway epithelial cells were increased following exposure to the high endotoxin LPS or CDE when compared to its low endotoxin-containing counterpart, these differences did not reach statistical significance ($p>0.05$).

Discussion

Our results indicate that following inhalation of LPS and CDE, which contain equal concentrations of endotoxin activity, similar physiologic and biological changes occur in otherwise healthy subjects. In addition, a similar percentage of study subjects developed specific symptoms following exposure to LPS and CDE at both high and low endotoxin concentrations, respectively. Moreover, the presence of respiratory symptoms, declines in airflow, and inflammation in the lower respiratory tract were more pronounced when study subjects were exposed to higher concentrations of endotoxin for both the LPS and CDE inhalation challenges. These results indicate that the concentration of endotoxin in the CDE strongly influences the physiologic and biological response and appears to be the principal component in grain dust causing acute airway injury.

Furthermore, our study results demonstrate that a dose-response relationship exists between the level of endotoxin present in the solutions administered by inhalation and the magnitude of the physiologic and biological changes measured in subjects exposed to these inhalants. Comparison of spirometric, BAL cellularity, BAL cytokine concentration, and serum measures of inflammation following exposure to the high and low endotoxin-containing LPS solutions resulted in greater declines in airflow and greater increases in BAL total cell, neutrophil, and cytokine concentrations following exposure to the high endotoxin LPS in comparison to the low endotoxin LPS solution. In a similar manner, the high endotoxin CDE solution produced greater

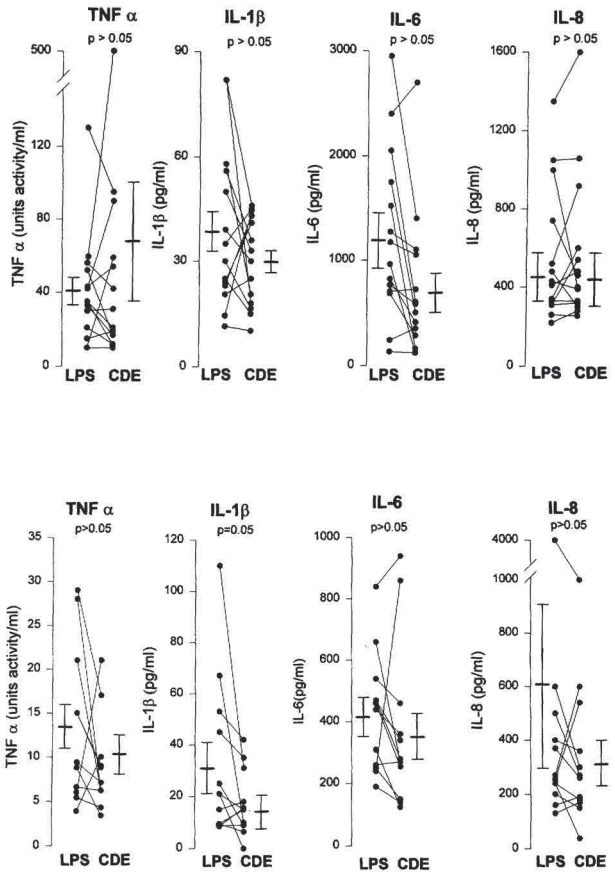


FIGURE 3. Cytokine concentrations in BAL fluid are shown for each subject following the high *(top)* and low *(bottom)* endotoxin LPS and CDE exposures. The group mean (±SEM) is represented for each exposure.

increases in measures of lung inflammation as suggested by the BAL fluid. However, higher concentrations of endotoxin in CDE were not associated with

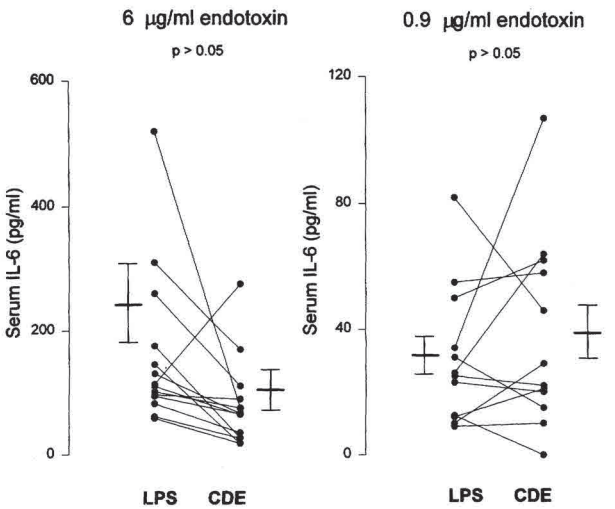


FIGURE 4. Serum IL-6 concentrations are shown for each subject following the high and low endotoxin LPS and CDE exposures. The group mean (±SEM) is represented for each exposure.

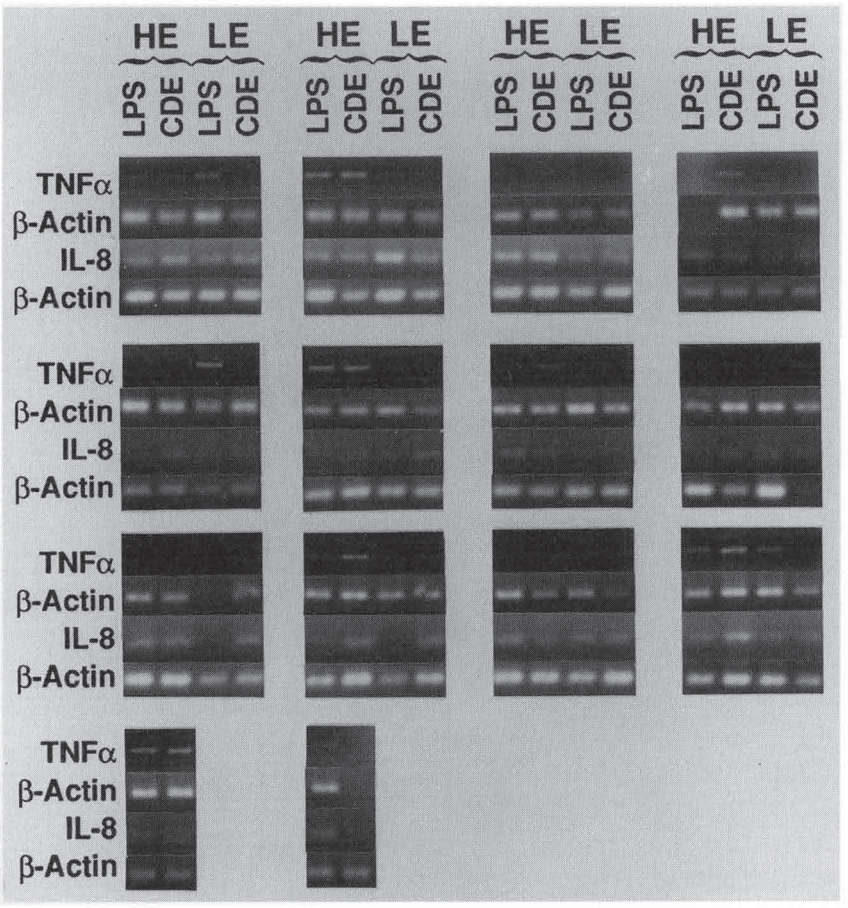


FIGURE 5. PCR products, generated by primers specific for TNF-α (BAL cells), IL-8 (airway epithelial cells), and β-actin are shown for each subject following exposure to the high endotoxin (HE) and low endotoxin (LE) LPS and CDE.

greater declines in airflow. One may speculate that the lack of a dose-response relationship between endotoxin concentration and the physiologic changes detected in the solutions is related to the presence of agents in grain dust, other than endotoxin, that may contribute to the development of airflow obstruction. However, at higher endotoxin concentrations, the physiologic effects of these additional bronchoconstricting agents to the development of airflow obstruction caused by endotoxin is of lower relative importance. Demonstration of a dose-response relationship between the endotoxin concentration in CDE and the magnitude of BAL and serum inflammatory cell and cytokine concentrations strongly suggests that endotoxin is the principal agent in CDE causing inflammatory cell recruitment and mediator release. In contrast, the role of agents in CDE, other than endotoxin, appears less important in initiating this response.

Interestingly, when differences were detected between LPS and CDE exposure, the LPS exposure consistently caused a greater effect than CDE. This observation suggests that agents in CDE, other than endotoxin, do not appear to cause any greater effect on the development of respiratory symptoms, airflow obstruction, inflammatory cell recruitment, or cytokine release than endotoxin alone. One explanation to account for the measured differences between LPS and CDE is that unlike their equivalent *in vitro* endotoxin activity, these agents may have slightly different *in vivo* biological activity. Unlike the CDE solution, the LPS solution contains a purified endotoxin derived from a single species of bacteria, *E coli.* In contrast, the endotoxins present in the CDE are heterogeneous, derived from the breakdown of bacterial organisms such as Pseudomonas, Enterobacter, and other taxa that contaminate this grain dust.[27] Therefore, these endotoxins could possess slightly different properties and produce differences in the host inflammatory response. Alternatively, substances in the extract, such as proteins, glucans, lectins, and phytotoxins, may alter the host's response to endotoxin or affect the bioavailability of the endotoxin due to protein binding. However, because the biological and physiologic changes associated with exposure to LPS and CDE were, in most cases, equivalent and not divergent, it would appear that these possible factors contributing to the

differences between exposures are of less importance than the overall observation of the similarity between LPS and CDE exposures.

One limitation of our study was that we compared the biological changes following exposure to LPS and CDE at a single time point, 4 h following inhalation. Whether BAL characteristics such as inflammatory cell numbers, cell types, and cytokine concentrations continue to be similar at various time points following exposure, and whether the inflammatory changes persist over the same period of time following exposure to each of these agents was not addressed in this study. Further studies looking at the kinetics of the inflammatory response to CDE and endotoxin at various time periods may be helpful in determining whether a substantial difference exists between the kinetics of inflammation for these two exposures.

Other investigators have studied agents in grain dust that may promote an inflammatory response or have toxic effects on cellular function. These substances include mycotoxins,[28] tannins,[29] lectins and lymphocyte mitogens,[30] and β(1,3)-D-glucan.[31] Although these agents may contribute to or modify the inflammatory effects resulting from short-term exposure to grain dust, our results provide evidence that endotoxin alone is sufficient to induce the early physiologic and biological changes following inhalation of grain dust.

The results of this study are consistent with our previous investigations examining the role of endotoxin in grain dust-induced airway disease. First, we have clearly demonstrated that among grain workers, the presence of respiratory symptoms and diminished measures of airflow are consistently related to the concentration of endotoxin in the work environment.[32] Second, following exposure to CDE, mice demonstrated a dose-response relationship between the concentration of endotoxin in the inhalant and the whole lung lavage concentration of total cells, neutrophils, and TNF-α.[27] However, in mice that are either genetically hyporesponsive to endotoxin or made tolerant to endotoxin, the inflammatory response to CDE in the lung was reduced significantly.[27] These findings suggest that the inflammatory response to grain dust is dependent on the host's ability to respond to endotoxin. Furthermore, by reducing the endotoxin activity in the CDE solution using three distinct methods of endotoxin removal, we have demonstrated that the resulting inflammatory response in the lungs of mice inhaling these endotoxin-depleted solutions was also reduced.[33] In aggregate, these observations in conjunction with the results of our current investigation demonstrate a strong association between endotoxin and the inflammatory response caused by grain dust inhalation.

In summary, this study provides further evidence to support the role of endotoxin as the primary mediator of airway inflammation in grain dust-induced lung disease. Monitoring exposure levels of airborne endotoxin in the workplace may be important, and consideration should be given to determining safe levels of exposure for individuals occupationally exposed to organic dust. Additionally, identification of biomarkers of exposure and host susceptibility factors to endotoxin may be equally important in determining whether an individual is at increased risk for the development of grain dust-induced airway disease.

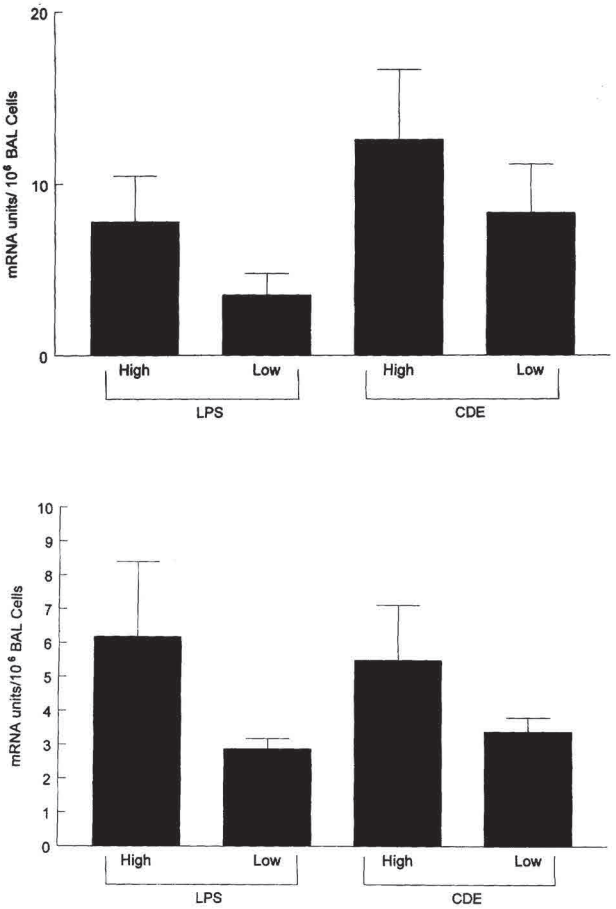


FIGURE 6. Densitometric analysis (mean±SEM) of IL-8 mRNA expression in airway epithelial cells *(top)* and TNF-α mRNA expression in BAL cells *(bottom)* are shown for each exposure.

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