Role of endotoxin in grain dust-induced lung inflammation in mice

PAUL J. JAGIELO, PETER S. THORNE, JEFFREY A. KERN, TIMOTHY J. QUINN, AND DAVID A. SCHWARTZ
Departments of Internal Medicine and Preventive Medicine and Environmental Health, The University of Iowa College of Medicine and the Department of Veterans Affairs, Iowa City, Iowa 52242–1081

Jagielo, Paul J., Peter S. Thorne, Jeffrey A. Kern, Timothy J. Quinn, and David A. Schwartz. Role of endotoxin in grain dust-induced lung inflammation in mice. *Am. J. Physiol.* 270 (*Lung Cell. Mol. Physiol.* 14): L1052–L1059, 1996.—To investigate the role of endotoxin in grain dust-induced airway inflammation, we reduced the endotoxin activity from extracts of corn dust (CDE), using three distinct methods, and determined the effect of endotoxin activity on the in vitro and in vivo inflammatory response to CDE. *Escherichia coli* lipopolysaccharide solution (LPS) and CDE solution were separated into >100-kDa and <100-kDa fractions by ultracentrifugation. Endotoxin activity was predominantly present in the >100-kDa fractions of the LPS and CDE solutions. Charged-membrane filtration of the >100-kDa fractions of LPS and CDE resulted in the reduction of endotoxin activity by 99.9 and 80%, respectively. Treatment of the >100-kDa fractions of LPS and CDE with polymyxin B-coated beads reduced the endotoxin activity by 96 and 89%, respectively. The untreated >100-kDa fractions of LPS and CDE caused significantly greater ($P < 0.01$) release of tumor necrosis factor-α (TNF-α) from THP-1 cells in vitro compared with its respective <100-kDa fraction or either of the treated (charged filter or polymyxin B) >100-kDa fractions. Similarly, mice exposed to either of the untreated >100-kDa fractions of LPS or CDE by inhalation developed significantly greater ($P < 0.01$) concentrations of lavage neutrophils and TNF-α in the lavage fluid compared with mice exposed to the respective <100-kDa fraction or either of the treated >100-kDa fractions. These results indicate that endotoxin is primarily responsible for the in vitro and in vivo inflammatory response to CDE.

endotoxin removal; airway inflammation; filtration; polymyxin B

INHALATION OF ORGANIC DUST is an occupational and environmental hazard, particularly for agricultural workers. In grain handlers, chronic grain dust exposure is associated with respiratory symptoms including cough, chronic bronchitis, chest tightness, and wheezing (7, 8, 12, 14, 18). Furthermore, exposure to grain dust results in the development of airflow obstruction (12) and bronchial hyperreactivity (23) and can lead to an accelerated longitudinal decline in measures of airflow (8, 18). A dose-response relationship exists between the level of grain dust exposure and lung function impairment (15).

To better understand the relationship between grain dust exposure and respiratory disease, models of grain dust exposure in both animals (3, 33) and humans (9, 10) have been developed. Extracts of grain dust derived from dust collected in the occupational setting have been used to study both the in vitro and in vivo inflammatory properties of grain dust (2, 6, 25, 27, 39). In previous studies using corn dust extract (CDE), non-atopic, nonasthmatic subjects without prior occupational exposure to grain dust developed acute airflow limitation and airway inflammation after inhalation of CDE (9, 10). These findings suggest that airway inflammation resulting from grain dust inhalation is mediated by nonallergic mechanisms.

One agent present in grain dust potentially responsible for grain dust-induced airway disease is endotoxin. Endotoxin is a component of the cell wall of Gram-negative bacteria which are contaminants of organic dusts. Endotoxin is readily measured in airborne samples of dust collected from grain elevators (11). Airborne endotoxin concentrations have been shown to be related to longitudinal declines in lung function in workers chronically exposed to grain dust (32). Furthermore, in inbred mice exposed to aerosolized CDE, the inflammatory response is dependent on the ability of the host to respond to endotoxin (33).

To further investigate the role of endotoxin in the inflammatory response to grain dust, we used three different methods for decreasing the endotoxin activity of CDE solution and measured the biological effect of these endotoxin-depleted solutions in both in vitro and in vivo test conditions. Previous investigators have described various methods of endotoxin activity removal from biological solutions (1, 16, 17, 24, 29, 36, 37). These methods include molecular mass exclusion, filtration removal through a positively charged membrane, and binding by polymyxin B resin. The objectives of the present study were *1*) to isolate the endotoxin activity in CDE solution through molecular-weight separation techniques; *2*) to remove endotoxin activity from CDE, using charged membrane filtration and polymyxin B resin binding; and *3*) to investigate relationships between the endotoxin activity in each of the molecular weight fractions of CDE solution and the in vitro and in vivo inflammatory response resulting from exposure to these solutions. A priori, we hypothesized that the inflammatory response to CDE was dependent on its endotoxin content and that decreasing the endotoxin activity in the CDE solution would reduce the inflammatory response.

METHODS

Chemicals. Lyophilized *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) was purchased from Sigma Chemical, St. Louis, MO. Corn dust used in this study was obtained from the air filtration system at an eastern Iowa grain handling

facility. Sterile, pyrogen-free water (pfw) and sterile, pyrogen-free normal saline (pfs) were purchased from Baxter Medical Laboratories, Deerfield, IL. Hanks' balanced salt solution (HBSS) was purchased from the University of Iowa tissue culture facility.

LPS and CDE preparation. LPS solution was prepared by mixing lyophilized *E. coli* LPS in pfw. CDE was prepared by mixing 3.0 g of dust in 30 ml of pfw (0.1% solution), vortexing for 2 min, and shaking for 1 h at 4°C. The mixture was centrifuged at 800 *g* for 20 min, and the supernatant solution was collected, resulting in the CDE. Both LPS and CDE solutions were filter sterilized through a 0.45-μm filter (Sterifil-D GV Filter Unit, Millipore, Bedford, MA) and then used immediately or stored at −70°C.

Endotoxin assay. Endotoxin concentrations were measured using the *Limulus* amebocyte lysate (LAL) assay (QCL-1000, Whittaker Bioproducts, Walkersville, MD). Lyophilized standard endotoxin from *E. coli* 0111:b4, chromogenic substrate, and chromogenic LAL preparations were reconstitiued with sterile pfw. Serial dilutions of CDE, LPS, and endotoxin standards were prepared with sterile pfw, and the assay was run in duplicate using sterile, pyrogen-free polystyrene microplates (Corning Glass Works, Corning, NY) at 37°C. Absorbance was measured at 405 nm, using a microplate reader (BT2000 MicroKinetics Reader, Bio-Tek Instruments, Palo Alto, CA). Change in absorbance relative to the assay reagent blank was calculated, and a standard curve was generated that ranged from 0.1 to 1.0 endotoxin units (EU) of National Institute of Standards and Technology traceable EC-5 standard endotoxin (10 EU = 1 ng endotoxin). Only assays in which standard curves had correlation coefficients >0.995 were accepted. For measurement of airborne endotoxin concentrations generated from the various LPS and CDE solutions during animal exposure studies, 0.30 m^3 of air was drawn from the exposure chamber through 47-mm binder-free glass microfiber filters (EPM-2000, Whatman International, Maidstone, UK) held within a 47-mm stainless steel inline air sampling filter holder (Gelman Sciences, Ann Arbor, MI). Air sampling filters were extracted with 10 ml of pfw at room temperature with gentle shaking for 1 h. This solution was then serially diluted with pfw and assayed for endotoxin concentration. Concentrations from four to six air samples were analyzed to determine a time-weighted exposure estimate.

Fractioning of LPS and CDE solutions by molecular weight. Ultrafiltration of LPS and CDE solutions was performed with the use of Centriprep concentrators (Amicon, Beverly, MA) that are capable of separating solutions by molecular weight exclusion. Unsonicated LPS and CDE solutions each underwent sequential ultrafiltration with Centriprep-3, Centriprep-10, Centriprep-30, Centriprep-50, and Centriprep-100 concentrators (3-, 10-, 30-, 50-, and 100-kDa filters, respectively). All filters were pretreated before their use by rinsing and spinning with 15 ml of pfw, 70% ethanol, and again with pfw. All samples underwent three spin periods at the *g*-forces and times specified by the manufacturer. Filters were used once and discarded. After initial ultrafiltration, the concentrated fraction (retentate) was brought to an equivalent volume as the filtered fraction (filtrate) with pfw before undergoing further filtration. Each of these filtered fractions of LPS and CDE solution was then assayed for endotoxin concentration by the LAL assay.

Separation of LPS and CDE for in vitro and in vivo experiments. LPS solution (7 μg/ml endotoxin activity) and CDE solution (2.5 μg/ml endotoxin activity) each were separated into >100-kDa and <100-kDa fractions, using Centriprep-100 concentrators. After separation, the >100-kDa fractions were then divided into three equal volumes. One aliquot underwent endotoxin depletion by charged nylon filtration. A second aliquot underwent endotoxin depletion by polymyxin B resin separation. The third aliquot remained untreated. Each of these solutions was lyophilized and then reconstituted to its original volume with HBSS. These solutions were then assayed for endotoxin activity before use in either in vitro and in vivo experiments.

Removal of endotoxin from LPS and CDE solutions. Removal of endotoxin from the >100-kDa fractions of both LPS and CDE solutions was performed, using two previously described methods. In the first method, each solution was filtered through a nonpyrogenic polycationic nylon filter (Zetapor SP 0.45-μm Pharmaceutical Grade Cartridge; Cuno, Meriden, CT). The Zetapor filter is capable of capturing 0.45-μm or larger particles due to pore-size exclusion. In addition, it is highly efficient at removing smaller, negatively charged particles, including endotoxin, through electrokinetic adsorption. The Zetapor filter was prewetted with 0.5 ml pfw. Ten milliliters of solution per filter were injected manually by syringe over 1 min; the average volume of solution reclaimed was 9.3 ml per filter. The filtrates were pooled, lyophilized, and reconstituted to their original volume with HBSS and assayed by LAL for endotoxin activity.

The second method of endotoxin removal was performed, using polymyxin B-coated agarose beads (P-1411, Sigma Chemical). The binding capacity of 1 ml of polymyxin B resin is 200–500 μg of endotoxin. Each of the high-molecular-weight fractions of LPS and CDE was pipetted into 50-ml polypropylene conical tubes, and 2 ml of resin were added to each tube. The tubes were mixed for 18 h on a rocker at 60 cycles/min. The polymyxin resin was removed by centrifugation (5 min at 800 *g*), and the supernatant was collected. The resulting solutions were pooled, lyophilized, reconstituted to their original volume with HBSS, and assayed by LAL for endotoxin.

Mice. Male, 6-wk-old, inbred mice (C3H/HeBFEJ) purchased from Jackson Laboratory (Bar Harbor, ME) were used in all exposure studies. They were housed in our institution's rodent vivarium, fed a normal diet (Formulab Chow no. 5008; PMI, Richmond, IN), provided water ad libitum, maintained on wood chip bedding (Northeastern Product, Warrensberg, NY), and used within 2 wk. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Iowa.

Exposure apparatus and exposure protocol. Mouse inhalation exposures to aerosols of LPS and CDE fractioned solutions were carried out as previously described (10). Exposures were performed in a 20-liter exposure chamber, using a PITT no. 1 nebulizer, with solutions for inhalation exposure supplied by a syringe pump. Each solution was delivered to the nebulizer at a fixed rate for each of the exposures to ensure equivalent volumes of solution were aerosolized.

In each experiment, groups of six mice underwent inhalation exposure to LPS or CDE solutions (untreated or endotoxin depleted) over 4 h. After exposure, animals were killed by cervical dislocation, the diaphragm was punctured to deflate the lungs, and whole lung lavage was performed.

Lung lavage. Mouse tracheas were isolated and cannulated with PE-90 tubing, and whole lung lavage was performed in situ. One-milliliter aliquots of pfs were infused into the trachea at 25-cmH_2O pressure head, collected, and the process was repeated six times. Lung lavage fluid was processed by our standard method (32).

In vitro endotoxin biological activity. To measure the in vitro cellular response to endotoxin in the various fractionated solutions of LPS and CDE, THP-1 cells (a human

monocytic leukemia cell line from American Type Culture Collection, Rockville, MD) were used (38). Cells (*passage 20–24*) were grown in pyrogen-free 10% RPMI medium in a 24-well cell culture plate (Costar, Cambridge, MA) at a density of 1×10^6 cells/well at 37°C, 5% CO_2. The cells were incubated with 0 or 10 μl of each of the fractionated LPS and CDE solutions noted above for 24 h. The cultures then underwent freeze-thaw cycling twice at −20°C, followed by centrifugation (2,500 *g*) for 5 min. The supernatants were collected and assayed for TNF-α.

TNF-α analysis. TNF-α bioactivity was measured, using the TNF-α-sensitive L929 mouse fibroblast cell assay (20). L929 cells were grown to confluence in a 96-well microtiter plate at a density of 4×10^4 cells/well. Fresh 5% Dulbecco's modified Eagle's medium (DMEM) containing actinomycin D (5 μg/ml) was added to each well, and each well was pulsed with 100 μl of sample or reference recombinant human TNF-α (Cetus, Emeryville, CA). Plates were incubated for 20 h (37°C, 5% CO_2), the medium was removed, and L929 cells were stained with 0.5% crystal violet in 20% methanol for 5 min. The optical density (OD) of the L929 cells was determined on a microplate reader (Bio-Tek EL311). Sample TNF-α values (units of activity/ml) were determined by comparing the sample OD to a standard curve generated with serial dilutions of reference TNF-α.

Statistical analysis. Two primary comparisons were made in this study: *1*) comparison of the in vitro TNF-α production of THP-1 cells exposed to the molecular fractions of LPS and CDE solutions before and after endotoxin removal, and *2*) comparison of the in vivo inflammatory response as assessed by both whole lung lavage fluid in mice exposed to these same solutions by inhalation. Statistical comparisons for continuous data were made using the Mann-Whitney *U*-test (31).

RESULTS

Identification of endotoxin in CDE fractions. Ultrafiltration was performed, using molecular weight-exclusion filters of varying sizes to separate the LPS and CDE solutions into distinct molecular weight fractions. As shown in Fig. 1*A*, the endotoxin component in the LPS solution was primarily localized to the >100-kDa fraction. This molecular weight fraction of LPS solution represented 89% of the total endotoxin measured in the intact LPS solution. A second, smaller component was identified in the 10- to 30-kDa fraction, containing 10% of the total endotoxin. The remaining fractions contained <1% of total endotoxin in solution. Separation of CDE by molecular weight produced similar results (Fig. 1*B*). Seventy-five percent of the total endotoxin activity was present in the >100-kDa fraction. The 10- to 30-kDa fraction contained ~25% of the total endotoxin, and <1% was measured in the remaining fractions of CDE.

Depletion of endotoxin from fractionated samples. Because the predominance of endotoxin was localized to the molecular fraction which was >100 kDa in both LPS and CDE solutions, depletion of endotoxin from these high-molecular-weight fractions was performed by filtration through a charged nylon filter and binding to polymyxin B-coated resin. Filtration of LPS solution through the charged filter resulted in 99.9% endotoxin removal (Fig. 2*A*). Similarly, the endotoxin concentration in the LPS solution was reduced by 96% after treatment with polymyxin B resin. The nylon-filtered,

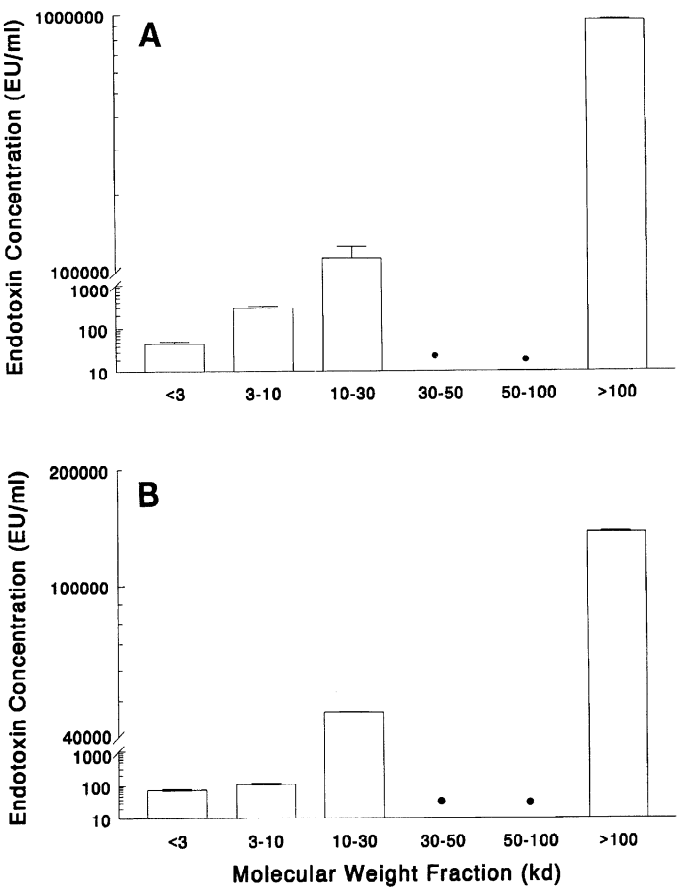

Fig. 1. Concentration of endotoxin is shown for each of the molecular weight fractions of lipopolysaccharide (LPS; *A*) and corn dust extract (CDE; *B*). Dots, endotoxin concentration <10 EU/ml; bars represent SE.

polymyxin B-treated, and the <100-kDa LPS fractions each had significantly less ($P < 0.01$) endotoxin activity compared with the untreated >100-kDa LPS fraction. Both of these methods of endotoxin removal were also effective in reducing the endotoxin from the CDE solution (Fig. 2*B*). The endotoxin concentration was reduced by 80% after nylon filtration and 89% after removal with polymyxin B. Endotoxin activity was significantly reduced ($P < 0.01$) in the nylon-filtered, the polymyxin-treated, and the <100-kDa CDE fractions compared with the untreated >100-kDa CDE fraction. In contrast to its efficiency at endotoxin removal in the LPS solution, nylon filtration was less effective at removing endotoxin from the CDE. However, the magnitude of reduction achieved by filtration of the >100-kDa fraction was sufficient to reduce the endotoxin concentration to levels comparable with those of the <100-kDa fraction.

In vitro biological response to fractioned samples. Incubation of THP-1 cells with the untreated >100-kDa LPS fraction resulted in a significant release of TNF-α, whereas no measurable TNF-α release was noted for the <100-kDa LPS fraction (Fig. 3*A*). Treatment of the >100-kDa LPS fraction by nylon filtration or with polymyxin B significantly reduced the ability of LPS to stimulate THP-1 cells to release TNF-α ($P <$ 0.01). Incubation of THP-1 cells with each of the CDE

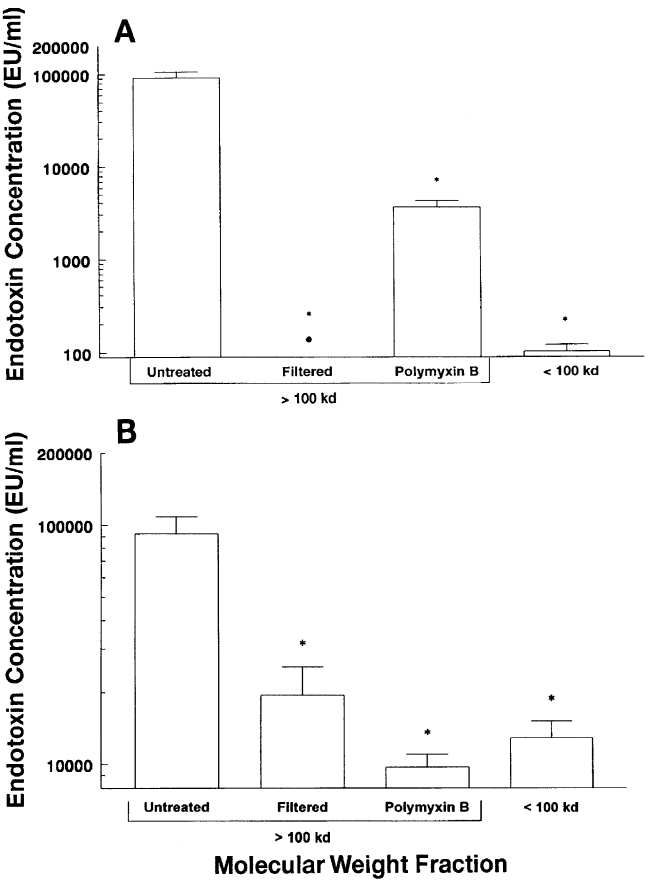

Fig. 2. Concentration of endotoxin for each of the fractions of LPS (*A*) and CDE (*B*): unfiltered >100-kDa fraction, nylon-filtered >100-kDa fraction, polymyxin B-treated >100-kDa fraction, and untreated <100-kDa fraction. Dot, endotoxin concentration <10 EU/ml; bars represent SE. *Statistically significant difference compared with respective untreated >100-kDa fraction; $P < 0.01$.

molecular weight fractions resulted in a pattern of TNF-α release similar to that seen with cells exposed to LPS (Fig. 3*A*). The untreated >100-kDa CDE fraction had a TNF-α stimulatory effect threefold greater than the <100-kDa fraction. Endotoxin reduction of CDE by filtration or treatment with polymyxin B resin resulted in significant reductions in the ability of the treated CDE to stimulate THP-1 cells to release TNF-α ($P < 0.01$).

In vivo biological response to fractionated samples. Mice exposed to the >100-kDa LPS fraction developed an increase in lavage cellularity (predominantly neutrophils) and an elevation in lavage fluid TNF-α (Fig. 4). In contrast, mice exposed to either the <100-kDa or the nylon-filtered >100-kDa fractions of LPS did not develop a neutrophilic inflammatory response, as the lavage fluid cells were predominantly macrophages and low levels of TNF-α were detected in the lavage fluid. Although neutrophils and TNF-α were present after inhalation of the high-molecular-weight fraction of LPS that was treated with polymyxin B, this inflammatory response was significantly less than what was observed after exposure to the untreated high-molecular-weight fraction of LPS. Interestingly, the magnitude of the in vivo response for each LPS fraction (Fig. 4) was consistent with its measured endotoxin activity (Fig. 2*A*), its in vitro response (Fig. 3*A*), and the measured airborne concentrations of endotoxin generated during the exposure (Fig. 4). Mice exposed to the >100-kDa CDE fraction (Fig. 5) developed a similar inflammatory response to that observed in mice exposed to the LPS >100-kDa fraction (Fig. 4). Lavage total cellularity (data not shown), neutrophil concentration, and TNF-α levels were all elevated in response to inhalation of the untreated >100-kDa fraction of CDE. In contrast, the inflammatory response measured in mice inhaling the <100-kDa CDE fraction was significantly less, with reductions in lavage concentrations of neutrophils and TNF-α ($P < 0.01$). Treatment of the >100-kDa CDE fraction by nylon filtration or with polymyxin B significantly reduced the inflammatory properties of the CDE, causing reductions in the concentration of lavage neutrophils and TNF-α compared with mice exposed to the untreated >100-kDa CDE ($P < 0.01$). In addition, lavage TNF-α concentrations appeared to correlate with the airborne concentrations of endotoxin measured during exposure to each of the CDE fractions. However, despite significant reductions in endo-

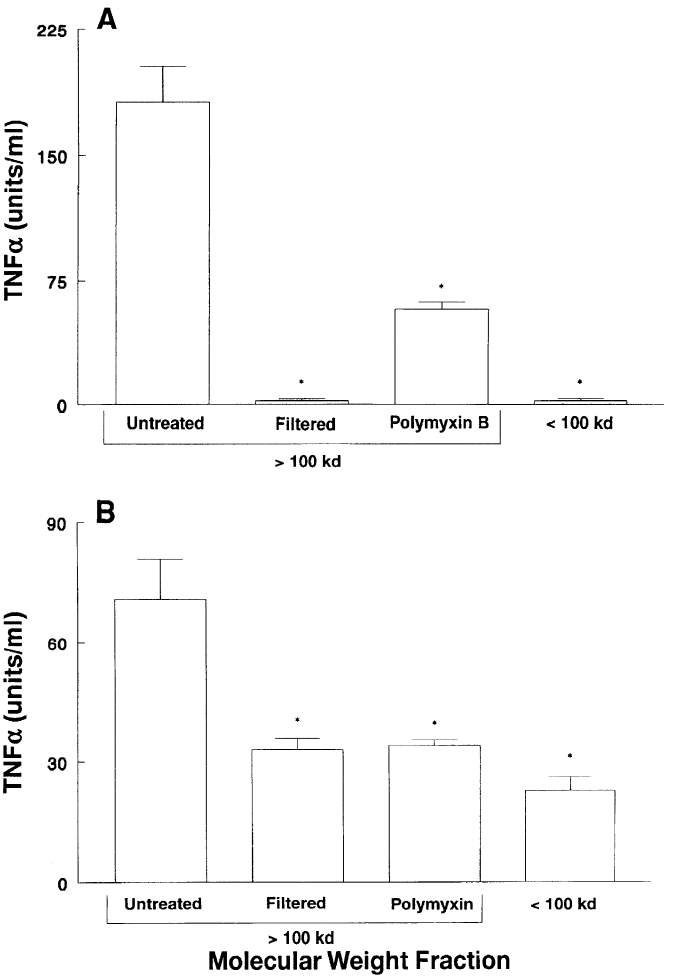

Fig. 3. Concentration of tumor necrosis factor-α (TNF-α) in cell lysate of THP-1 cells for each fraction of LPS (*A*) and CDE (*B*) solution: unfiltered >100-kDa fraction, nylon-filtered >100-kDa fraction, polymyxin B-treated >100-kDa fraction, and untreated <100-kDa fraction. Bars represent SE. *Statistically significant difference compared with respective untreated >100-kDa fraction; $P < 0.01$.

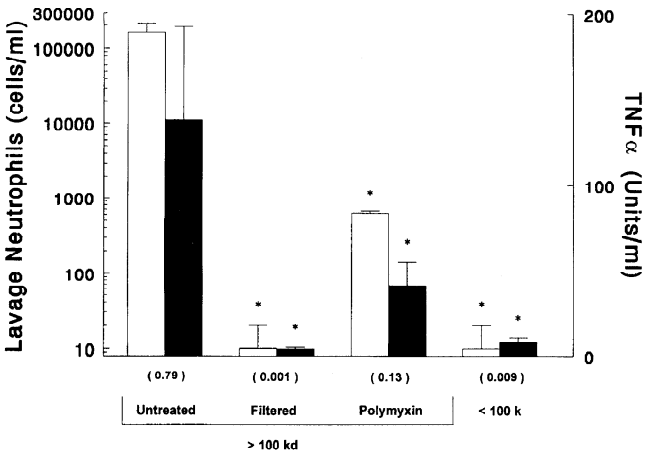

Fig. 4. Lung lavage mean concentrations of neutrophils (open bars) and TNF-α (solid bars) are shown after inhalation exposure (6 mice per exposure) to each fraction of LPS solution. Airborne endotoxin concentrations (μg/m^3) measured during exposure to each fraction are shown in parentheses. Bars represent SE. *Statistically significant difference compared with respective untreated >100-kDa fraction; $P < 0.01$.

toxin activity from the filtered, polymyxin B-treated, and <100-kDa CDE fractions (Fig. 2*B*), mice exposed to these endotoxin-depleted fractions had elevations in lavage neutrophils which were not observed in the endotoxin-depleted LPS fractions having similar endotoxin activity.

To determine whether reconstitution of endotoxin activity from endotoxin-depleted <100-kDa CDE results in a restoration of its in vivo inflammatory activity, a separate experiment was carried out in which LPS was added back to the <100-kDa CDE fraction to achieve an endotoxin activity equivalent to the >100-kDa CDE fraction. Groups of six mice were exposed to the >100-kDa CDE (endotoxin activity 5.8 μg/ml), <100-kDa CDE (0.1 μg/ml), and <100-kDa CDE reconstituted with LPS (5.8 μg/ml). As shown in Fig. 6, the concentrations of lavage neutrophils and

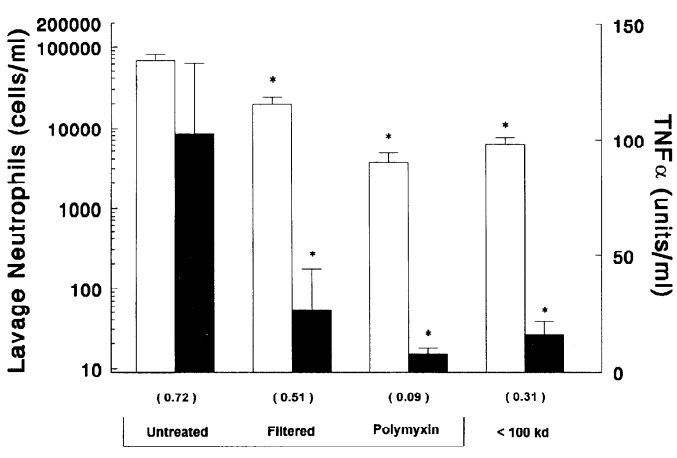

Fig. 5. Lung lavage mean concentrations of neutrophils (open bars) and TNF-α (solid bars) after inhalation exposure (6 mice per exposure) to each fraction of CDE solution. Airborne endotoxin concentrations (μg/m^3) measured during exposure to each fraction are shown in parentheses. Bars represent SE. *Statistically significant difference compared with respective untreated >100-kDa fraction; $P < 0.01$.

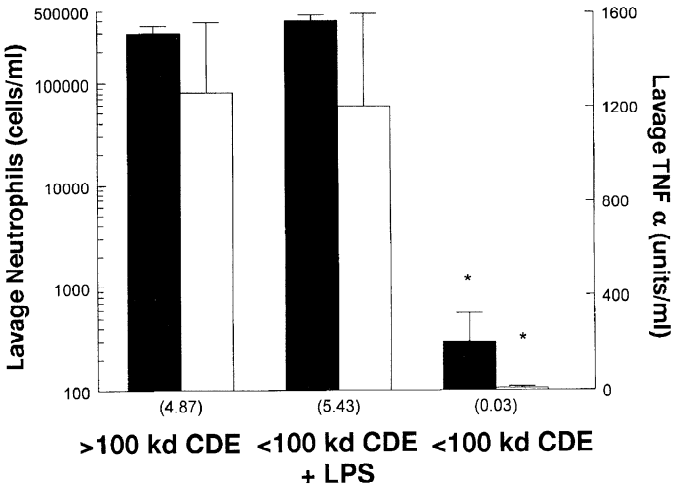

Fig. 6. Lung lavage mean concentrations of neutrophils (open bars) and TNF-α (solid bars) after inhalation exposure (6 mice per exposure) to >100-kDa CDE, <100-kDa CDE reconstituted with LPS, and <100-kDa CDE. Airborne endotoxin concentrations (μg/m^3) measured during exposure to each fraction are shown in parenthesis. Bars represent SE. *Statistically significant difference compared with <100-kDa CDE fraction; $P < 0.01$.

TNF-α were similar for mice exposed to the >100-kDa CDE fraction and <100-kDa CDE fraction reconstituted with LPS, and these were significantly greater than the endotoxin-depleted <100-kDa CDE fraction.

DISCUSSION

Our results indicate that endotoxin is primarily responsible for the in vitro and in vivo inflammatory response to CDE solution. Using three distinct methods to decrease the endotoxin activity in CDE solution, we demonstrated that when the endotoxin activity in the CDE was reduced, the resulting inflammatory response was also reduced. After separation of the CDE solution into >100-kDa and <100-kDa molecular mass fractions, the predominance of endotoxin activity was measured in the higher molecular weight fraction. This finding is consistent with previous observations (36) demonstrating that in an aqueous environment free of divalent cations, endotoxin exists primarily as micelles of 300,000 to 1,000,000 molecular weight. In vitro and in vivo studies demonstrated a significantly greater inflammatory response to the higher molecular weight fraction than to the lower molecular weight fraction, with significantly greater release of TNF-α by THP-1 cells exposed to the >100-kDa CDE fraction, and significantly greater lung inflammation measured by lavage in mice exposed to the >100-kDa CDE fraction. Furthermore, when the >100-kDa CDE fraction underwent charged membrane filtration or treatment with polymyxin B, the endotoxin activity was significantly decreased. Coinciding with this decrease in endotoxin activity, there was a significant reduction in the in vitro and in vivo inflammatory response compared with the untreated >100-kDa CDE solution. When endotoxin activity was restored to the endotoxin-depleted <100-kDa CDE fraction, the magnitude of the inflammatory response returned to levels similar to the undepleted

fraction. In all instances, the magnitude of inflammatory activity of each fractioned CDE solution correlated with the endotoxin activity present in solution.

These results complement our previous findings (33) in which we demonstrated that the inflammatory response to CDE is dependent on the host's ability to respond to endotoxin. Using an inherent model of endotoxin hyporesponsiveness, we demonstrated that endotoxin-resistant mice (C3H/HeJ) developed significantly less lung inflammation after inhalation of CDE than control mice (C3H/HeBFEJ), which are genetically susceptible to the toxic effects of endotoxin. Furthermore, using a model of acquired endotoxin hyporesponsiveness, endotoxin-tolerant mice developed significantly less lung inflammation compared with nontolerant control mice. Thus by either reducing the host's ability to respond to endotoxin or reducing the endotoxin activity in the CDE solution, we have demonstrated a strong association between endotoxin and lung inflammation in the setting of acute CDE inhalation. These results support our overall hypothesis that endotoxin is the principal component in grain dust responsible for inducing lung inflammation in grain dust-induced airway disease.

We considered the possibility that other substances in CDE in addition to endotoxin may have been removed by the methods employed to deplete endotoxin activity from CDE. CDE represents the water-soluble and insoluble components (<0.45 μm) in grain dust. Grain dust is a complex mixture of vegetable particles, microbes and their products, insect fragments, animal proteins and excreta, feed additives, and pesticides (13). It is conceivable that these foreign proteins and chemicals could be capable of inducing inflammation. In the present study, however, we have consistently demonstrated an association between endotoxin activity reduction from CDE and a corresponding reduction in the magnitude of the inflammatory response, using three completely different methods of endotoxin removal. It is unlikely that the same substance or substances were removed from CDE by all three methods of endotoxin removal unless they were bound to endotoxin. Furthermore, by demonstrating that the reconstitution of endotoxin activity to depleted fractions of CDE results in the restoration of its proinflammatory activity, these observations strongly support the role of endotoxin as a primary agent in promoting lung inflammation.

Extracts of grain dust have been shown to be biologically active in vitro and in vivo. Extracts of grain dust have been shown to activate both the classical and alternate complement pathways (25, 27, 40), stimulate release of histamine from mast cells (6), stimulate alveolar macrophages to release cytokines (unpublished observations), produce neutrophilic alveolitis in animals (33), cause respiratory symptoms (10) and acute declines in airflow (10), and attract neutrophils in the upper and lower respiratory tract in human subjects exposed to the aerosolized extract (9). Inhalation of CDE results in increased concentrations of total cells, neutrophils, interleukin (IL)-1β, IL-1 RA, IL-6, IL-8, and TNF-α in bronchoalveolar lavage fluid 4 h after exposure (9). Moreover, it appears that the alveolar macrophage is the principal cell responsible for the production of these inflammatory cytokines after grain dust exposure (unpublished observations). Importantly, in human subjects, inhalation of LPS or CDE produces similar physiological effects (10). These findings, together with those of our present study, demonstrate the role of endotoxin as a potentially important mediator of airway inflammation.

Interestingly, despite significant reductions in endotoxin activity from CDE, a neutrophilic inflammatory response in the lavage fluid was observed in mice exposed to the endotoxin-depleted CDE fractions that was not observed in mice exposed to endotoxin-depleted LPS fractions. This observation suggests that at low endotoxin levels, other components in CDE apart from endotoxin may play a role in attracting neutrophils into the airways after exposure to grain dust. Von Essen et al. (39) demonstrated in vitro that extracts of various grain dusts have the ability to attract neutrophils directly through complement activation as well as indirectly via activation of alveolar macrophages. Furthermore, whereas depletion of endotoxin from grain sorghum extract resulted in the reduction in the capacity of macrophages to release chemotactic activity for neutrophils, the ability of the endotoxin-depleted grain sorghum extract to activate complement was preserved. Tannins in grain dust have been shown to activate complement (34) and may stimulate the production of neutrophil chemotactic factors from alveolar macrophages (30). In addition, mycotoxins which may be biologically active are present in significant quantities in grain dust (28). Bacterial components other than endotoxin, such as prokaryotic DNA, have been shown to possess immune-stimulating properties (20). Thus these or other as-yet-unidentified agent(s) in grain dust may be capable of attracting neutrophils into the airways through direct or indirect mechanisms. Taken together, these results suggest that the inflammatory response to grain dust is, at least in part, due to endotoxin contamination, although other components in grain dust may play an additional role in causing acute inflammation.

Previous studies of cotton dust exposure support the relationship between endotoxin exposure and changes in lung function due to exposure to organic dust. In studies of experimental cotton dust exposure in humans, there appears to be a consistent correlation between the respirable airborne endotoxin concentration and airflow obstruction (5). Washing cotton may remove up to 95% of the endotoxin in the dust (22). Furthermore, in experimental cotton dust exposure, inhalation of washed cotton dust produces significantly less decrements in airflow compared with exposure to unwashed cotton dust (13).

Although a substantial reduction in the endotoxin concentration from the CDE solution was accomplished by either charged membrane filtration or by polymyxin B resin binding, total depletion of endotoxin was not achieved. Endotoxin removal from the LPS solutions by

charged membrane filtration or polymyxin B resin binding appeared to be more complete than removal of endotoxin from CDE solution. Proteins and other positively charged molecules in the CDE solution that may bind endotoxin may have prevented its removal by either process. Conditions such as solution pH above 8.5, presence of soluble organics, and solute content, especially proteins, may decrease the efficacy of endotoxin removal (17). Despite these limitations, both methods of endotoxin removal were able to reduce the endotoxin concentration in the CDE solutions to a level sufficient to alter the biological effects observed in vitro and in vivo.

Although the purpose of this study was not to compare the in vitro and in vivo inflammatory response between similar fractions of LPS and CDE, one interesting observation should be noted. Although the >100-kDa fractions of LPS and CDE had equivalent endotoxin activity (Fig. 2, *A* and *B*), the release of TNF-α from THP-1 cells was greater for LPS than CDE (Fig. 3, *A* and *B*). Furthermore, there also was a greater neutrophilic response to the >100-kDa LPS compared with >100-kDa fraction of CDE. However, the lavage TNF-α concentrations were similar after both exposures. These results suggest that for an equivalent concentration of endotoxin activity, LPS may cause greater inflammatory response than CDE. One possible explanation to account for this difference is that agents in CDE other than endotoxin may cause a positive LAL reaction but may not have biological activity in vitro and in vivo. Alternatively, assuming the LAL assay is truly measuring endotoxin, there may be component(s) in CDE which act to modulate the in vitro or in vivo inflammatory effects of endotoxin.

In the inhalation studies performed, one may observe a difference in the magnitude of the inflammatory response to the >100-kDa fraction of CDE in Fig. 5 compared with Fig. 6. This discrepancy is explained by differences in the aerosol exposure generation between these experiments. As shown in Fig. 5, the aerosolized levels of endotoxin generated from the CDE were significantly lower (0.72 μg/m^3) compared with the experiment performed in Fig. 6 (4.87 μg/m^3). This would account for the lower concentrations of neutrophils and TNF-α in the lavage fluid in mice exposed to lower airborne endotoxin levels. This observation is consistent with our previous findings demonstrating a dose-response relationship between the airborne level of endotoxin and the magnitude of the inflammatory response in mice after exposure to CDE (33).

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. Identifying endotoxin as the principal agent in the inflammatory response in grain dust-induced lung disease has important implications. First, exposure levels of airborne endotoxin in the workplace should be closely monitored, and safe levels of exposure should be identified to prevent disease in exposed individuals. Second, biomarkers of exposure and host factors responsible for causing increased susceptibility to endotoxin-induced lung inflammation may be important to identify to minimize the risk of chronic airway disease in individuals exposed to grain dust. Finally, insight into therapeutic means of reducing endotoxin-induced lung inflammation should be explored, since grain dust exposure is often unavoidable.

This study was supported by grants from the National Institute of Occupational Safety and Health of the Centers for Disease Control (U07-CCU-706145), the National Institute of Environmental Health Sciences (ES-O6537), the General Clinical Research Centers Program (RR-00059), National Center for Research Resources, National Institutes of Health Fellowship Training Grant HL-07638, and the Department of Veterans Affairs (Merit Review).

Address for reprint requests: D. A. Schwartz, Pulmonary Diseases, Critical Care, Occupational Medicine Division, Dept. of Internal Medicine, The Univ. of Iowa College of Medicine, Iowa City, Iowa 52242–1081.

Received 3 May 1995; accepted in final form 15 January 1996.

REFERENCES

1. **Aida, Y., and M. J. Pabst.** Removal of endotoxin from protein solutions by phase separation using Triton X-114. *J. Immunol. Methods* 132: 191–195, 1990.
2. **Alam, R., D. M. Lewis, and S. A. Olenchock.** Activation of guinea pig lymphocytes and mast cells by grain-dust extract. *J. Allergy Clin. Immunol.* 81: 598–604, 1988.
3. **Armanious, M., D. Cotton, J. Wright, J. Dosman, and I. Carr.** Grain dust and alveolar macrophages: an experimental study of the effects of grain dust on the mouse lung. *J. Pathol.* 136: 265–272, 1982.
5. **Castellan, R. M., S. A. Olenchock, K. B. Kinsley, and J. L. Hankinson.** Inhaled endotoxin and decreased spirometric values. *N. Engl. J. Med.* 317: 605–610, 1987.
6. **Chan-Yeung, M., H. Chan, H. Salari, R. Wall, and K. S. Tse.** Grain-dust extract induced direct release of mediators from human lung tissue. *J. Allergy Clin. Immunol.* 80: 279–284, 1987.
7. **Chan-Yeung, M., M. Schulzer, L. MacLean, E. Dorken, and S. Grzybowski.** Epidemiologic health survey of grain elevator workers in British Columbia. *Am. Rev. Respir. Dis.* 121: 329–338, 1980.
8. **Chan-Yeung, M., M. Schulzer. L. MacLean, E. Dorken, F. Tan, S. Lam, D. Enarson, and S. Grzybowski.** A follow-up study of the grain elevator workers in the Port of Vancouver. *Arch. Environ. Health* 36: 75–81, 1981.
9. **Clapp, W. D., S. Becker, J. Quay, J. L. Watt, P. S. Thorne, X. L. Frees, X. Zhang, H. S. Koren, C. R. Lux, and D. A. Schwartz.** Grain dust-induced airflow obstruction, and inflammation of the lower respiratory tract. *Am. J. Respir. Crit. Care Med.* 150: 611–617, 1994.
10. **Clapp, W. D., P. S. Thorne, K. L. Frees, X. Zhang, C. R. Lux, and D. A. Schwartz.** The acute effects of inhalation of grain dust extract and endotoxin on upper and lower airways. *Chest* 104: 825–830, 1993.
11. **Del, A. J., M. A. Godshali, and M. S. Palmgren.** Gram-negative bacterial endotoxins in grain elevator dusts. *Am. Ind. Hyg. Assoc. J.* 45: 336–339, 1984.
12. **DoPico, G. A., W. Reddan, A. Tsiatis, M. E. Peters, and J. Rankin.** Epidemiologic study of clinical and physiologic parameters in grain handlers of Northern United States. *Am. Rev. Respir. Dis.* 130: 759–765, 1984.
13. **Dosman, J. A., and D. J. Cotton.** Grain dust and health. III. Environmental factors. *Ann. Int. Med.* 89: 420–421, 1978.
14. **Dosman, J. A., D. J. Cotton, B. L. Graham,, K. Y. R. Li, F. Froh, and G. D. Barnett.** Chronic bronchitis and decreased forced expiratory flow rates in lifetime nonsmoking grain workers. *Am. Rev. Respir. Dis.* 121: 11–16, 1980.
15. **Enarson, D. A., S. Vedal, and M. Chan-Yeung.** Rapid decline in FEV_1 in grain handlers. *Am. Rev. Respir. Dis.* 132: 814–817, 1985.
16. **Gerba, C. P., and K. Hou.** Endotoxin removal by charge-modified filters. *Appl. Environ. Microbiol.* 50: 1375–1377, 1985.

17. **Hou, K., C. P. Gerba, S. M. Goyal, and K. S. Zerda.** Capture of latex beads, bacteria, endotoxin, and viruses by charge-modified filters. *Appl. Environ. Microbiol.* 40: 892–896, 1980.
18. **Huy, T., K. D. Schipper, M. Chan-Yeung, and S. M. Kennedy.** Grain dust and lung function. Dose-response relationships. *Am. Rev. Respir. Dis.* 144: 1314–1321, 1991.
19. **James, A. L., W. O. C. M. Cookson, G. Buters, S. Lewis, G. Ryan, R. Hockey, and A. W. Musk.** Symptoms and longitudinal changes in lung function in young seasonal grain handlers. *Br. J. Ind. Med.* 43: 587–591, 1986.
20. **Krieg, A. M., A.-K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman.** CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature Lond.* 374: 546–549, 1995.
21. **Leeper-Woodford, S. K., P. D. Carey, K. Byrne, J. K. Jenkins, B. J. Fisher, C. Blocher, H. J. Sugerman, and A. A. Fowler.** Tumor necrosis factor: alpha and beta subunits appear in the circulation during onset of sepsis-induced lung injury. *Am. Rev. Respir. Dis.* 143: 1076–1082, 1991.
22. **Merchant, J. A., J. C. Lumsden, K. H. Kilburn, V. H. Germino, J. D. Hamilton, W. S. Lynn, J. Burd, and D. Baucom.** Preprocessing cotton to prevent byssinosis. *Br. J. Ind. Med.* 30: 237–247, 1973.
23. **Mink, J. T., J. W. Gerrard, D. W. Cockcroft, D. J. Cotton, and J. A. Dosman.** Increased bronchial reactivity to inhaled histamine in nonsmoking grain workers with normal lung function. *Chest* 77: 28–31, 1980.
24. **Morrison, D. C., and D. M. Jacobs.** Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry* 13: 813–818, 1976.
25. **Olenchock, S. A., and P. C. Major.** Immunological studies of grain dust. *J. Environ. Pathol. Toxicol.* 2: 9–22, 1979.
26. **Olenchock, S. A., J. C. Mull, and W. G. Jones.** Endotoxins in cotton: washing effects and size distribution. *Am. J. Ind. Med.* 4: 515–521, 1983.
27. **Olenchock, S. A., J. C. Mull, P. C. Major, M. J. Peach, M. E. Gladish, and G. Taylor.** In vitro activation of the alternative pathway of complement by settled grain dust. *J. Allergy Clin. Immunol.* 62: 295–300, 1978.
28. **Palmgren, J. S., and L. S. Lee.** Separation of mycotoxin-containing sources in grain dust and determination of their mycotoxic potential. *Environ. Health Perspect.* 66: 105–108, 1986.
29. **Ribi, E., R. L. Anacker, R. Brown, W. T. Haskins, B. Malmgren, K. C. Milner, and J. A. Rudbach.** Reaction of endotoxin and surfactants. I. Physical and biological properties of endotoxin treated with sodium deoxycholate. *J. Bacteriol.* 92: 1493–1509, 1966.
30. **Rohrbach, M. S., T. Kreofsky, R. A. Rolstad, and J. A. Russell.** Tannin-mediated secretion of a neutrophil chemotactic factor from alveolar macrophages. *Am. Rev. Respir. Dis.* 139: 39–45, 1989.
31. **Rosner, B.** *Fundamentals of Biostatistics* (3rd ed.). Boston, MA: PWS-Kent Publishing, 1990.
32. **Schwartz, D. A., D. L. Landas, D. L. Lassise, L. F. Burmeister, G. W. Hunninghake, and J. A. Merchant.** Airway injury in swine confinement workers. *Ann. Intern. Med.* 116: 630–635, 1992.
33. **Schwartz, D. A., P. S. Thorne, P. J. Jagielo, G. E. White, S. A. Bleuer, and K. L. Frees.** Endotoxin responsiveness and grain dust-induced inflammation in the lower respiratory tract. *Am. J. Physiol.* 267 (*Lung Cell. Mol. Physiol.* 11): L609–L617, 1994.
34. **Skea, D. L., D. McAvoy, and I. Broder.** Grain dust contains tannin-like material which fixes complement. *Environ. Res.* 46: 181–189, 1988.
35. **Smid, T., D. Heederik, R. Houba, and P. H. Quanjer.** Dust- and endotoxin-related respiratory effects in the animal feed industry. *Am. Rev. Respir. Dis.* 146: 1474–1479, 1992.
36. **Sweadner, K. J., M. Forte, and L. L. Nelsen.** Filtration removal of endotoxin (pyrogens) in solution in different states of aggregation. *Appl. Environ. Microbiol.* 34: 382–385, 1977.
37. **Talmadge, K. W., and C. J. Siebert.** Efficient endotoxin removal with a new sanitizable affinity column: affi-prep polymyxin. *J. Chromatogr.* 476: 175–185, 1989.
38. **Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada.** Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer* 26: 171–176, 1980.
39. **Von Essen, S. G., R. A. Robbins, A. B. Thompson, R. F. Ertl, J. Linder, and S. Rennard.** Mechanisms of neutrophil recruitment to the lung by grain dust exposure. *Am. Rev. Respir. Dis.* 138: 921–927, 1988.
40. **Wirtz, G. H., S. S. Westfall, and B. J. Davidson.** Interactions of complement with an extract of airborne spring wheat dust. *J. Toxicol. Environ. Health* 14: 511–523, 1984.