Grain dust-induced lung inflammation is reduced by *Rhodobacter sphaeroides* diphosphoryl lipid A

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Jagielo, Paul J., Timothy J. Quinn, Nilofer Qureshi, and David A. Schwartz. Grain dust-induced lung inflammation is reduced by *Rhodobacter sphaeroides* diphosphoryl lipid A. *Am. J. Physiol.* 274 (*Lung Cell. Mol. Physiol.* 18): L26–L31, 1998.—To further determine the importance of endotoxin in grain dust-induced inflammation of the lower respiratory tract, we evaluated the efficacy of pentaacylated diphosphoryl lipid A derived from the lipopolysaccharide of *Rhodobacter sphaeroides* (RsDPLA) as a partial agonist of grain dust-induced airway inflammation. RsDPLA is a relatively inactive compound compared with lipid A derived from *Escherichia coli* (LPS) and has been demonstrated to act as a partial agonist of LPS-induced inflammation. To assess the potential stimulatory effect of RsDPLA in relation to LPS, we incubated THP-1 cells with RsDPLA (0.001–100 μg/ml), LPS (0.02 μg endotoxin activity/ml), or corn dust extract (CDE; 0.02 μg endotoxin activity/ml). Incubation with RsDPLA revealed a tumor necrosis factor (TNF)-α stimulatory effect at 100 μg/ml. In contrast, incubation with LPS or CDE resulted in TNF-α release at 0.02 μg/ml. Pretreatment of THP-1 cells with varying concentrations of RsDPLA before incubation with LPS or CDE (0.02 μg endotoxin activity/ml) resulted in a dose-dependent reduction in the LPS- or CDE-induced release of TNF-α with concentrations of RsDPLA of up to 10 μg/ml but not at 100 μg/ml. To further understand the role of endotoxin in grain dust-induced airway inflammation, we utilized the unique LPS inhibitory property of RsDPLA to determine the inflammatory response to inhaled CDE in mice in the presence of RsDPLA. Ten micrograms of RsDPLA intratracheally did not cause a significant inflammatory response compared with intratracheal saline. However, pretreatment of mice with 10 μg of RsDPLA intratracheally before exposure to CDE (5.4 and 0.2 μg/m^3) or LPS (7.2 and 0.28 μg/m^3) resulted in significant reductions in the lung lavage concentrations of total cells, neutrophils, and specific proinflammatory cytokines compared with mice pretreated with sterile saline. These results confirm the LPS-inhibitory effect of RsDPLA and support the role of endotoxin as the principal agent in grain dust causing airway inflammation.

endotoxin; lipopolysaccharide inhibition; asthma

OCCUPATIONAL AND ENVIRONMENTAL exposure to grain dust may cause a variety of acute respiratory symptoms, including chest tightness, wheezing, dyspnea, and transient declines in airflow (6). Epidemiologic studies in grain workers have demonstrated that chronic exposure to grain dust may lead to chronic respiratory symptoms, including chronic bronchitis, and progressive, irreversible airflow obstruction (1). Prior studies suggest an association between endotoxin and the physiological and inflammatory response to grain dust. In grain handlers, work-related respiratory symptoms were found to be strongly associated with the concentration of endotoxin in the bioaerosol (15). Inhalation of endotoxin and corn dust extract (CDE) produces similar symptoms, equivalent changes in airflow, and similar increases in bronchoalveolar lavage inflammatory cells and inflammatory cytokines (5).

To further investigate the relative importance of endotoxin in grain dust-induced airway inflammation, we have employed methods that attempted to remove endotoxin from CDE and demonstrated a relationship between the concentration of endotoxin in CDE and the magnitude of inflammation in the lung (4). However, these methods were limited by the inability to completely remove endotoxin from CDE and by the possibility of removal of other components in CDE that may promote lung inflammation. In this investigation, we propose to examine the relationship between endotoxin and grain dust-induced lung inflammation by specifically inhibiting endotoxin-mediated inflammatory events. Previous investigations have shown that the pentaacylated diphosphoryl lipid A derived from the lipopolysaccharide of *Rhodobacter sphaeroides* (RsDPLA) is a relatively inactive compound compared with lipid A derived from *Escherichia coli* (LPS; see Ref. 16). Moreover, it appears that RsDPLA acts as a partial agonist of LPS-induced inflammation (7, 17). In this investigation, we utilized the unique LPS inhibitory property of RsDPLA to determine the relative stimulatory effect of CDE in vitro and the inflammatory response in the lungs of mice after exposure to CDE in the presence of an endotoxin inhibitor. A priori, we hypothesize that the inflammatory response to CDE will be diminished in the presence of RsDPLA.

METHODS

Chemicals. Lyophilized *E. coli* 0111:B4 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 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 HBSS. CDE was prepared by mixing 3.0 g of dust in 30 ml of HBSS (0.1% solution) by methods previously described (4). Both LPS and CDE solutions were filter sterilized through a 0.22-μm filter (Acrocap Low Protein Binding Filter; Gelman Sciences, Ann Arbor, MI) and then were used immediately or were stored at −70°C.

RsDPLA preparation. R. sphaeroides (ATCC 17023) was grown photoheterotrophically for 12–14 days and was harvested by centrifugation. LPS was extracted from *R. sphaeroides* by the phenol-chloroform-petroleum ether method of

Galanos et al. (3). RsDPLA was prepared by treating *R. sphaeroides* LPS with 0.02 M sodium acetate (pH 2.5) for 70 min at 100°C (10). Crude RsDPLA was dissolved in chloroform-methanol and was purified by DEAE-cellulose column chromatography. RsDPLA was eluted with a linear ammonium acetate gradient and was characterized by analytical thin-layer chromatography, gas-liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy (9). The RsDPLA was in the free acid form, was solubilized in 0.5% triethylamine salt, and was further diluted in saline before use in the experiments.

Endotoxin assay. Endotoxin concentrations were measured in the LPS and CDE solutions using the *Limulus* amebocyte lysate (LAL) assay (QCL-1000; Whittaker Bioproducts, Walkersville, MD). Measurement of airborne endotoxin concentrations generated from the various LPS and CDE solutions during animal exposure studies was carried out using methods previously described (14). The sensitivity of the LAL for aerosolized LPS is 0.25 ng/m^3.

Mice. Male 6-wk-old inbred mice (C3Heb/FeJ) 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, Warrensburg, 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. A series of four mouse inhalation exposure experiments was performed to aerosols of LPS and CDE, the methods of which we have previously described (14). Exposures were performed in a 20-liter exposure chamber with a PITT no. 1 nebulizer with solutions for inhalation exposure supplied by a syringe pump. In each experiment, groups of 20 mice underwent inhalation exposure to LPS or CDE solutions over 4 h. Thirty minutes before inhalation challenge, each mouse underwent general anesthesia (Metofane; Pitman-Moore, Mundelein, IL) followed by instillation of equivalent volumes (40 μl) of RsDPLA (10 μg) or PFS into the hypopharynx through a 24-gauge Jelco intravenous catheter (Johnson and Johnson, Arlington, TX). This dose of RsDPLA was chosen after performing dose-response experiments with mice that demonstrated that 10 μg of RsDPLA do not cause changes in lung lavage cellularity compared with PFS. After exposure, animals were killed by cervical dislocation, the diaphragm was punctured to deflate the lungs, and whole lung lavage was performed.

Lung lavage. Mice tracheae 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 and collected, and the process was repeated six times. Lung lavage fluid was processed by our standard method (13).

In vitro endotoxin biological activity. To measure the in vitro cellular response to RsDPLA, LPS, and CDE, THP-1 cells (American Type Culture Collection, Rockville, MD), a human monocytic leukemia cell line, were used (18). Cells (*passages 20-24*) were grown in pyrogen-free 10% RPMI 1640 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 in 1 ml of medium. For dose-response curve experiments, the cells were incubated with RsDPLA at varying concentrations (0, 0.001, 0.01, 0.1, 1.0, 10, and 100 μg/ml) in HBSS. For coincubation studies with LPS or CDE, RsDPLA (0–100 μg/ml) was introduced 30 min before treatment with LPS or CDE (endotoxin concentration 0.02 μg/ml). After 24 h, the cultures then underwent freeze-thaw cycling two times at −20°C followed by centrifugation (2,500 *g*) for 5 min. The supernatants were collected and assayed for tumor necrosis factor (TNF)-α.

Cytokine analyses. Commercially available kits (R&D Systems, Minneapolis, MN) were used to determine concentrations of murine interleukin (IL)-1β, IL-6, macrophage inflammatory protein-2 (MIP-2), TNF-α, and human TNF-α. In all cases, a monoclonal antibody was used as a capture reagent in a standard sandwich enzyme-linked immunosorbent assay (ELISA). The assays were performed according to the manufacturer's instructions, and standard curves were derived using known concentrations of the recombinant specific cytokine supplied by the manufacturer. All assays were performed using the standard diluent supplied in the ELISA kit.

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 LPS and CDE solutions after pretreatment with RsDPLA and *2*) comparison of the in vivo inflammatory response as assessed by both whole lung lavage fluid in mice exposed to LPS or CDE after pretreatment with RsDPLA or sterile saline intratracheally. Statistical comparisons for continuous data were made using the Mann-Whitney *U*-test (11).

RESULTS

The in vitro stimulatory effect of RsDPLA on THP-1 cells was determined by performing dose-response experiments in which THP-1 cells were incubated with increasing concentrations of RsDPLA (0.001–100 μg/ml) for 24 h. The supernatant fluid was assayed for TNF-α (Fig. 1*A*). At relatively low concentrations of RsDPLA (≤10 μg/ml), very little TNF-α stimulatory effect was observed compared with higher concentrations of RsDPLA (100 μg/ml), which caused substantially greater stimulatory release of TNF-α in a dose-dependent fashion. Although higher concentrations of RsDPLA caused a stimulatory effect on THP-1 cells, this effect was achieved at a considerably higher concentration compared with LPS, which stimulates TNF-α release at concentrations as low as 0.01 μg/ml (Fig. 1*B*).

Because of its relative nonstimulatory effect at low concentrations, further studies were carried out to determine whether RsDPLA could display inhibitory properties when incubated with cells before LPS or CDE stimulation. Incubation of THP-1 cells with LPS (Fig. 2*A*) or CDE (Fig. 2*B*) alone (endotoxin activity 0.02 μg/ml) resulted in the stimulation of TNF-α production and release from these cells. When THP-1 cells were pretreated with 10-fold increasing concentrations of RsDPLA (0.001–100 μg/ml) before stimulation with LPS or CDE, there was a similar pattern in the magnitude of TNF-α release from THP-1 cells for each concentration of RsDPLA. At lower concentrations of RsDPLA (0.001–1 μg/ml), there was a dose-dependent reduction in LPS and CDE stimulatory release of TNF-α, which was maximal in both instances at 1 μg/ml. However, this inhibitory effect was not apparent at 100 μg/ml, where RsDPLA stimulates THP-1 cells to produce and release TNF-α (Fig. 1*A*).

RsDPLA was tested for its in vivo ability to stimulate lung inflammation. Mice underwent intratracheal instillation of RsDPLA at doses of 0.1, 1, 10, and 100 μg or sterile saline, and these doses of RsDPLA (except 100

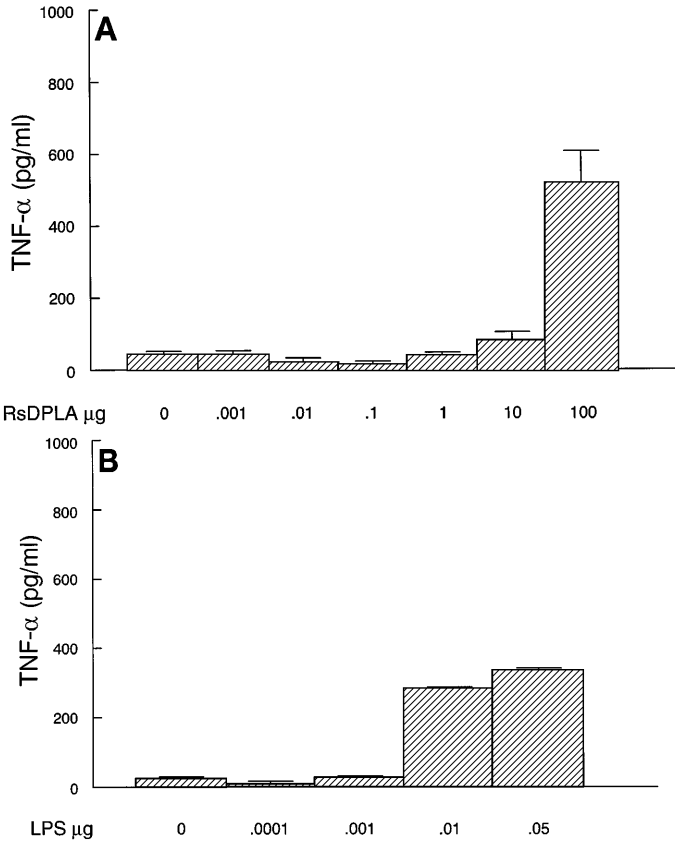

Fig. 1. Mean concentration of tumor necrosis factor (TNF)-α in the cell lysate of THP-1 cells is presented after incubation with varying concentrations of pentaacylated diphosphoryl lipid A derived from the lipopolysaccharide of *Rhodobacter sphaeroides* (RsDPLA; *A*) or lipid A derived from *Escherichia coli* (LPS; *B*). Three samples were processed at each dosage. Error bars are the SE.

μg) were not found to stimulate neutrophil recruitment in the lung (data not shown). Because of the lack of neutrophil recruitment in the lung lavage fluid after intratracheal instillation of RsDPLA at 10 μg, this dose was chosen for subsequent experiments to determine whether RsDPLA had inhibitory properties when given intratracheally before inhalation exposure to LPS or CDE.

After intratracheal instillation of RsDPLA or HBSS, groups of 10 mice underwent inhalation challenge to LPS or CDE at various airborne endotoxin levels. At high airborne levels of endotoxin (LPS: 7.2 μg/m^3; CDE: 5.4 μg/m^3), mice pretreated with HBSS developed increasing concentrations of total cells, predominantly neutrophils, as well as elevated concentrations of TNF-α, IL-1β, IL-6, and MIP-2 in lung lavage fluid after exposure to both LPS (Fig. 3*A*) and CDE (Fig. 3*B*). In mice pretreated with RsDPLA, statistically significant reductions in both total cell, neutrophil, IL-1β, and IL-6 concentrations were observed after exposure to LPS or CDE compared with HBSS-pretreated mice exposed to the same bioaerosols ($P < 0.05$ for all comparisons made). At high airborne levels of endotoxin, no differences in lung lavage TNF-α or MIP-2 were detected between RsDPLA- and HBSS-treated mice after exposure to either LPS or CDE.

Mice pretreated with HBSS followed by exposure to low airborne levels of endotoxin (LPS: 0.28 μg/m^3; CDE: 0.2 μg/m^3) developed increased lung lavage concentrations of total cells, neutrophils, TNF-α, IL-1β, IL-6, and MIP-2 (Fig. 4, *A* and *B*), but the increases in cellularity and cytokine concentrations were not as great compared with the high endotoxin exposures (Fig. 3, *A* and *B*). After exposure to LPS, mice pretreated with RsDPLA demonstrated significant reductions in lavage concentrations of total cells, neutrophils, TNF-α, IL-6, and MIP-2 compared with control mice pretreated with HBSS. After exposure to CDE, mice pretreated with RsDPLA also demonstrated significant reductions in lavage concentrations of total cells, neutrophils, TNF-α, and MIP-2, but no differences in lavage concentrations of IL-1β or IL-6 were detected between treatment groups.

DISCUSSION

Our study results confirm previous observations that RsDPLA is less potent than LPS at stimulating TNF-α release but is capable of inhibiting LPS-mediated TNF-α release (17). Likewise, RsDPLA is capable of inhibiting CDE-mediated production and release of TNF-α, suggesting a similar mechanism by which LPS and CDE

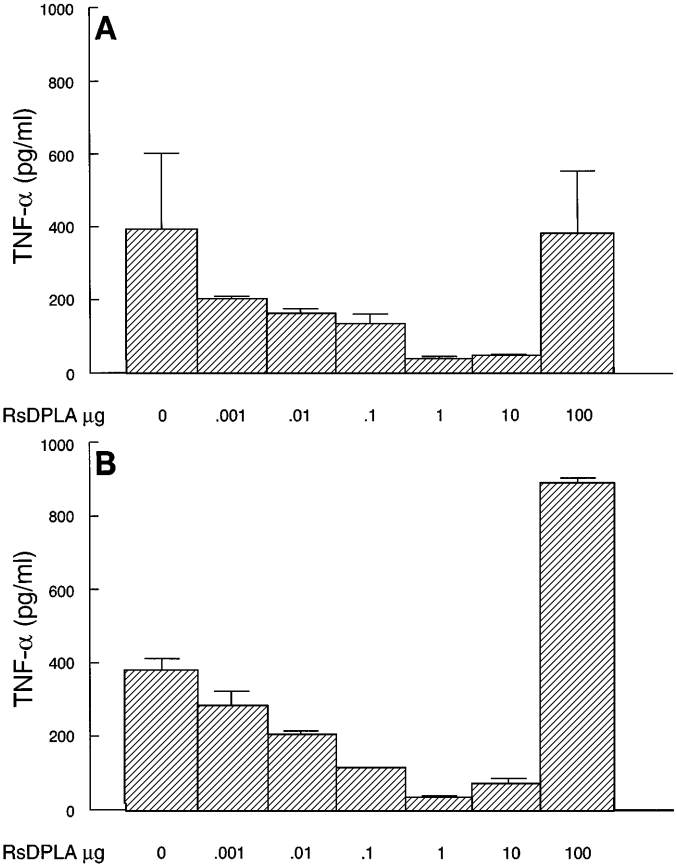

Fig. 2. Mean concentration of TNF-α in the cell lysate of THP-1 cells is shown for cells incubated with LPS (*A*) and corn dust extract (CDE; *B*) alone or pretreated with varying concentrations of RsDPLA. Concentration of endotoxin in LPS and CDE solutions was 0.02 μg/ml. Three samples were processed at each dosage. Error bars are the SE.

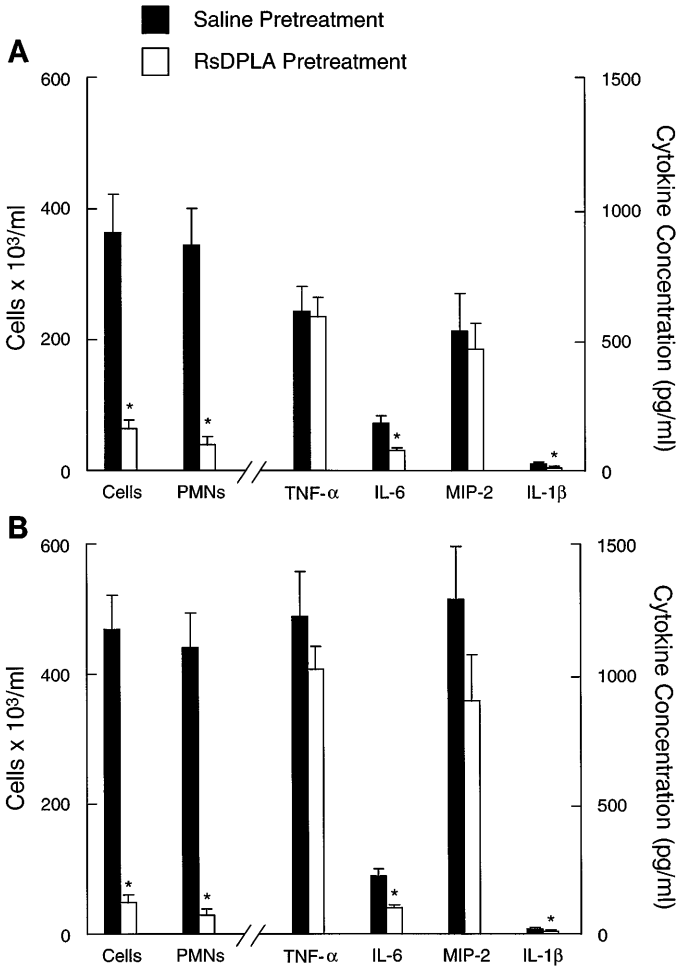

Fig. 3. Lung lavage mean concentrations of total cells, polymorphonuclear neutrophils (PMNs), TNF-α, interleukin (IL)-6, macrophage inflammatory protein (MIP)-2, and IL-1β are presented after inhalation exposure to LPS (*A*) and CDE (*B*) for mice pretreated intratracheally with RsDPLA or sterile saline. Airborne endotoxin concentrations measured during each exposure: LPS, 7.2 μg/m^3; CDE, 5.4 μg/m^3. Error bars are the SE. *$P < 0.02$.

stimulate THP-1 cells to produce and release TNF-α. Furthermore, when administered to mice intratracheally at a dose of 10 μg, RsDPLA alone does not cause significant lung inflammation. However, when 10 μg of RsDPLA were administered before inhalation of either LPS or CDE, this dose was capable of inhibiting LPS- and CDE-mediated lung inflammation, again suggesting a common mechanism by which LPS and CDE cause lung inflammation. Our results suggest that CDE causes lung inflammation through endotoxin-mediated inflammatory events, which are inhibited by RsDPLA.

The results of this study add further support to the growing evidence that endotoxin is one of the primary agents in grain dust causing airway inflammation. In previous epidemiologic studies, we have found that the concentration of endotoxin in the bioaerosol is strongly associated with respiratory symptoms, airflow obstruction, and longitudinal declines in airflow among grain handlers and other farmers (12, 15). In addition, we have previously investigated the relationship between endotoxin and grain dust-induced airway disease using a murine model of acute grain dust inhalation challenge. We have demonstrated a clear dose-response relationship between the concentration of endotoxin in the CDE bioaerosol and the magnitude of lung inflammation and cytokine release in lung lavage fluid (14). A second approach involved the determination of whether host susceptibility to endotoxin affected the inflammatory response to CDE (14). Utilizing strains of mice that are genetically endotoxin hyporesponsive (C3H/HeJ) or endotoxin sensitive (C3Heb/FeJ), we previously demonstrated that when exposed to an identical CDE exposure, mice that are genetically hyporesponsive to endotoxin develop significantly less lung lavage inflammatory cells and cytokines compared with endotoxin-sensitive mice. Furthermore, when endotoxin-sensitive mice were made endotoxin tolerant (acquired endotoxin hyporesponsiveness) through serial injections with LPS, these mice developed significantly less airway inflammation compared with control mice after the same CDE exposure. A third approach examining the relationship

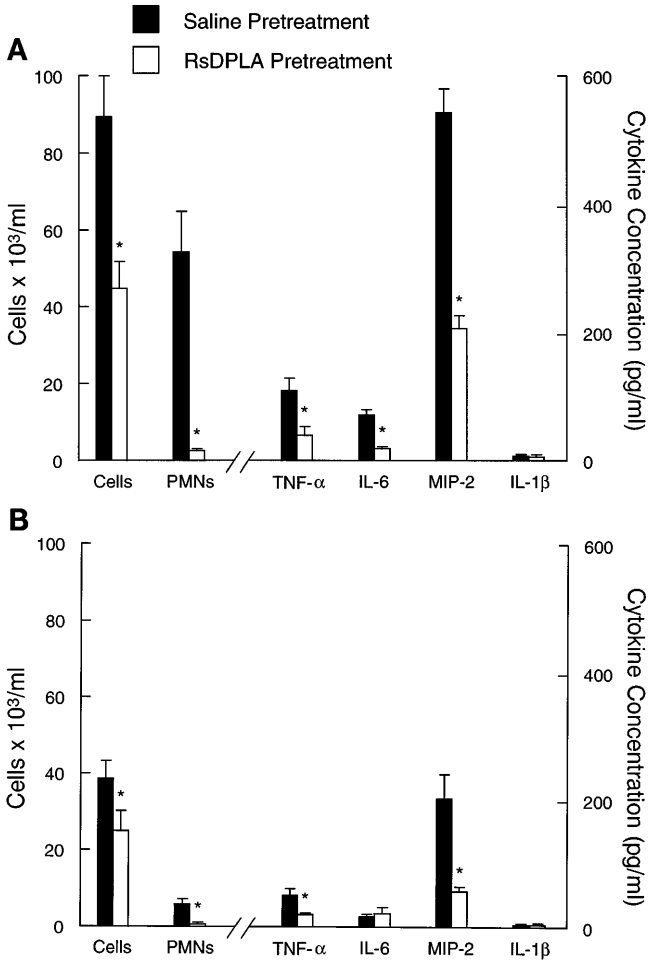

Fig. 4. Lung lavage mean concentrations of total cells, PMNs, TNF-α, IL-6, MIP-2, and IL-1β are presented after inhalation exposure to LPS (*A*) and CDE (*B*) for mice pretreated intratracheally with RsDPLA or sterile saline. Note that the scale of the *y*-axis is different from Fig. 3. Airborne endotoxin concentrations measured during each exposure: LPS, 0.28 μg/m^3; CDE, 0.2 μg/m^3. Error bars are the SE. *$P < 0.03$.

between endotoxin and grain dust-induced lung inflammation involved determining whether endotoxin removal from CDE altered the magnitude of CDE-induced lung inflammation. In these experiments, we demonstrated that, using three different methods of endotoxin removal from biological solutions, the magnitude of the inflammatory response to CDE was related to the endotoxin concentration in the CDE (4). The results from these previous investigations support the findings from our current study. In the present investigation, by utilizing the unique properties of RsDPLA that act as a specific inhibitor of endotoxin, we have demonstrated that the inflammatory response to CDE is substantially reduced when this agent is given intratracheally before exposure to CDE. In aggregate, we have demonstrated convincing evidence linking endotoxin with the acute inflammatory response to CDE by demonstrating a dose-response relationship between endotoxin concentration in the CDE bioaerosol and the magnitude of lung inflammation, by altering the host's ability to respond to endotoxin, by altering the concentration of endotoxin in CDE, and by selectively inhibiting endotoxin-mediated inflammatory events.

The results of our study are the first to demonstrate the ability of RsDPLA to inhibit endotoxin-mediated lung inflammation using an inhalational model of acute lung inflammation. Previous investigations have demonstrated that RsDPLA is capable of blocking the induction of TNF-α from a macrophage cell line treated with LPS in a concentration-dependent manner (17). Pretreatment of mice with RsDPLA intravenously blocked the rise in serum TNF-α normally observed in mice after intravenous injection with LPS (8). Furthermore, RsDPLA was protective in preventing endotoxin-mediated death in mice, possibly due to induction of early endotoxin tolerance (19). Our present investigation supports the role of RsDPLA as an inhibitor of CDE-induced lung inflammation by acting as a competitive agonist of endotoxin-mediated induction of specific proinflammatory cytokines in vitro. The mechanism by which RsDPLA acts to reduce lung inflammation due to grain dust in our study is not entirely clear but may be due to direct inhibition of endotoxin binding to cell receptors in the lung or possibly secondary to the induction of early endotoxin tolerance.

Cytokines undoubtedly play a major role in the inflammatory response to inhaled grain dust. In humans and mice, we have demonstrated that TNF-α, IL-6, IL-8 (humans), and MIP-2 (mice) are released within hours of the exposure and may persist for up to 48 h after the inhalation challenge (2). Interestingly, mice pretreated with RsDPLA followed by inhalation challenge with either LPS or CDE at high endotoxin concentrations did not develop significant reductions in lung lavage concentrations of TNF-α despite reductions in lavage cellularity, IL-1β, and IL-6 levels (Fig. 3, *A* and *B*). In contrast, TNF-α and MIP-2 concentrations in lung lavage fluid were significantly reduced in mice pretreated with RsDPLA exposed to lower concentrations of endotoxin in LPS or CDE (Fig. 4, *A* and *B*). This observation may be attributed to the possibility that the airborne levels of endotoxin in the high-endotoxin LPS or CDE exposures exceeded the inhibitory capacity of RsDPLA used in the study. In addition, because RsDPLA was administered intratracheally and not by aerosolization, the distribution of RsDPLA in the lung may have differed between these routes of administration, affecting the efficacy of RsDPLA in inhibiting LPS- or CDE-induced TNF-α and MIP-2 release at the higher endotoxin concentrations. Finally, the complex combination of cytokine gradients may play an important role in recruitment of neutrophils after different types of inhalation challenges.

In conclusion, our results confirm prior observations demonstrating the relative nontoxic properties of RsDPLA, including intratracheal lung administration. In addition, we have demonstrated that RsDPLA inhibits grain dust-induced lung inflammation, supporting the hypothesis that endotoxin is the primary component in grain dust responsible for the development of airway inflammation.

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