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Inhibition of LPS-induced airway hyperresponsiveness and airway inflammation by LPS antagonists

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Schwartz, David A., William J. Christ, Steven R. Kleeberger, and Christine L. Wohlford-Lenane. Inhibition of LPS-induced airway hyperresponsiveness and airway inflammation by LPS antagonists. *Am J Physiol Lung Cell Mol Physiol* 280: L771–L778, 2001.—To determine whether the inflammatory effects of inhaled endotoxin could be prevented, we pretreated mice with synthetic competitive antagonists (975, 1044, and 1287) for lipopolysaccharide (LPS) before a LPS inhalation challenge. In preliminary studies, we found that these LPS antagonists did not act as agonists in vitro (THP-1 cells) or in vivo (after intratracheal instillation of 10 μg) and that these compounds (at least 1 μg/ml) effectively antagonized the release of tumor necrosis factor-α by LPS-stimulated THP-1 cells. Pretreatment of mice with 10 μg of either 1044 or 1287 resulted in a decrease in the LPS-induced airway hyperreactivity. Moreover, pretreatment of mice with 10 μg of 975, 1044, or 1287 resulted in significant reductions in LPS-induced lung lavage fluid concentrations of total cells, neutrophils, and specific proinflammatory cytokines compared with mice pretreated with sterile saline. Using residual oil fly ash to induce airway inflammation, we found that the action of the LPS antagonists was specific to LPS-induced airway disease. These results suggest that LPS antagonists may be an effective and potentially safe treatment for endotoxin-induced airway disease.

asthma; endotoxin; lung inflammation; lipopolysaccharide

ASTHMA, A DISORDER CHARACTERIZED by airway hyperreactivity and reversible inflammation of the airways, is an increasing cause of morbidity and mortality in this country, particularly among children (22). The cause for its rising severity is unknown, although factors as disparate as poor access to medical care (3) and allergic responses to cockroach antigens (27) have been cited. Factors that induce or perpetuate the inflammatory response have an adverse effect on asthma outcomes. Although environmental allergens have been associated with increased asthma severity and frequency of exacerbations, the role of other inhaled agents is less clearly defined. Endotoxin, a cell wall component of gram-negative bacteria that is a lipopolysaccharide (LPS), is ubiquitous in the environment and is often present in high concentrations in organic dusts (29) as well as in air pollution (2). Given the potent inflammatory effects of endotoxin, it is logical to consider the role of this agent in the development and exacerbation of asthma.

In fact, several lines of evidence indicate that endotoxin is an important component of the bioaerosol that contributes to airway inflammation and airflow obstruction. First, the concentration of inhaled endotoxin in the bioaerosol is strongly associated with the development of acute decrements in airflow among cotton workers (20), swine confinement workers (14, 32), and poultry workers (36). The concentration of endotoxin in the bioaerosol is the most important occupational exposure associated with the development (30) and progression (32) of airway disease in agricultural workers. Second, physiologically, inhaled endotoxin (24, 28), grain dust (10), or cotton dust (6) can cause airflow obstruction in naive or previously unexposed subjects. Naive, healthy study subjects challenged with dust from animal confinement buildings develop airflow obstruction and an increase in the serum concentration of neutrophils and interleukin (IL)-6, all of which are most strongly associated with the concentration of endotoxin (not dust) in the bioaerosol (38). Third, our previous exposure-response studies have shown that inhaled grain dust and endotoxin produce similar physiological and biological effects in humans (19) and mice (31), the concentration of endotoxin in grain dust plays an important role in the acute biological response to grain dust in humans (19) and mice (31), and genetic or acquired hyporesponsiveness to endotoxin substantially reduces the biological response to grain dust in mice (31). Finally, recent reports have indicated that the concentration of endotoxin in the domestic setting is related to the clinical severity of asthma (23–25) and is a biologically important component of air pollution particulate matter (2, 4).

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To determine whether the inflammatory effects of inhaled endotoxin could be prevented, we pretreated mice with a competitive antagonist for LPS [*Rhodobacter sphaeroides* diphosphoryl lipid A (RsDPLA)] and found that it reduced the inflammatory response to inhaled grain dust in mice (18). In the series of studies presented here, we pursued these findings with RsDPLA by using the unique LPS-inhibitory properties of synthetic LPS antagonists (9) to determine whether these agents are capable of inhibiting endotoxin-induced airway hyperreactivity and airway inflammation. A priori, we hypothesized that the physiological and inflammatory responses to endotoxin would be diminished in the presence of these LPS antagonists.

METHODS

Protocol. To determine whether the synthetic LPS antagonists could prevent the airway response to inhaled LPS, we conducted three types of experiments. First, we used a human monocytic leukemia cell line (THP-1 cells) to determine whether the LPS antagonists could prevent the THP-1 cells from releasing tumor necrosis factor (TNF)-α when stimulated with LPS. THP-1 cells were chosen for the in vitro assay because macrophages play a prominent role in LPS-induced airway disease (37). Second, we intratracheally instilled the LPS antagonists in mice to determine whether the LPS antagonists caused airway inflammation. Finally, we pretreated mice intratracheally with either saline or one of the LPS antagonists, exposed the mice to a 4-h LPS inhalation challenge, and measured the effect of the LPS antagonist on LPS-induced airway hyperreactivity and airway inflammation. The specificity of action was evaluated by determining the effect of pretreatment with 1287 on the airway response to intratracheal instillation of residual oil fly ash (ROFA).

Chemicals. Lyophilized *Escherichia coli* 0111:B4 LPS was purchased from Sigma (St. Louis, MO). 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 (Iowa City, IA) Tissue Culture Facility.

LPS preparation. LPS solution was prepared by mixing lyophilized *E. coli* LPS in HBSS. The LPS solution was filter sterilized through a 0.22-μm filter (Acrocap low protein binding filter, Gelman Sciences, Ann Arbor, MI) and was then used immediately or stored at −70°C.

LPS antagonists. The LPS antagonists used in this investigation included 975, 1044, and 1287 and were supplied by the Eisai Research Institute (Andover, MA). These specific antagonists are stable and have demonstrated potent antagonism of LPS-mediated cellular activation in a variety of in vitro and in vivo assays (7–9). 975 is a triether-stabilized analog of the *Rhodobacter capsulatus* lipid A and has both high hydrolytic stability and high antagonist activity. 1044 is a diether-stabilized analog of the *R. sphaeroides* lipid A that demonstrates a high antagonist activity profile. However, 1044 can be degraded by loss of a 2′-olefin-bearing side chain, generating some agonistic by-products. 1287 resolves the problems of 1044; it is highly stable and very water soluble and has a high antagonist activity profile.

Endotoxin assay. Endotoxin concentrations were measured in the LPS solutions with the *Limulus* amebocyte lysate (LAL) assay (QCL-1000, Whittaker BioProducts, Walkersville, MD). Measurement of airborne endotoxin concentrations generated from the various LPS solutions during animal exposure studies were also carried out with methods previously described (31). The sensitivity of the LAL assay for aerosolized LPS was 0.25 ng/m^3.

In vitro endotoxin biological activity. THP-1 cells (American Type Culture Collection, Manassas, VA), a human monocytic leukemia cell line, was used to measure the in vitro cellular response to the LPS antagonists and LPS. Cells (*passages 20–24*) were grown in 1 ml of 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 and in 5% CO_2. For the dose-response curve experiments, the cells were incubated with LPS antagonists at varying concentrations (0, 0.01, 0.1, 1.0, 10, or 100 μg/ml) in HBSS. For coincubation studies with LPS, LPS antagonists (0–100 μg/ml) were introduced 30 min before treatment with LPS (endotoxin concentration 0.02 μg/ml). After 24 h, the cultures underwent freeze-thaw cycling twice at −20°C followed by

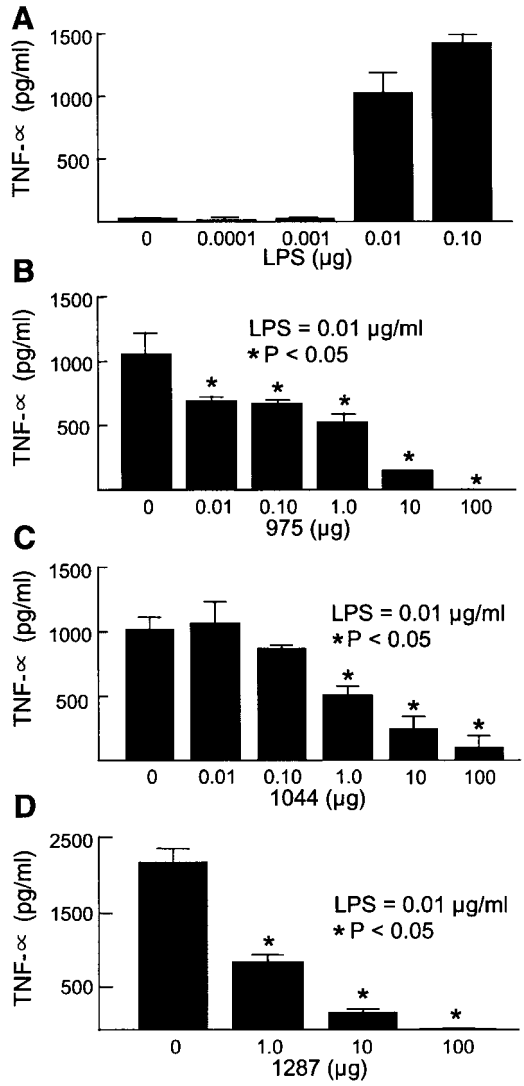

Fig. 1. Concentration of tumor necrosis factor (TNF)-α in the cell lysate of THP-1 cells is shown for cells incubated with lipopolysaccharide (LPS) alone (*A*) or for cells pretreated with varying concentrations of 975 (*B*), 1044 (*C*), or 1287 (*D*) and then cultured with LPS. Three samples were processed at each dose. Values are means ± SE. *P* values compare LPS with increasing doses of LPS antagonist with LPS alone.

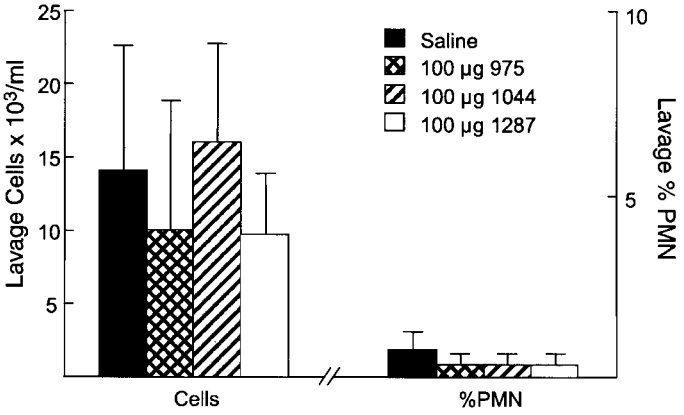

Fig. 2. Lung lavage fluid concentration of total cells and percentage of polymorphonuclear neutrophils (PMNs) 4 h after intratracheal instillation of saline or the 3 LPS antagonists (975, 1044, and 1287). Values are means ± SE.

centrifugation (2,500 *g*) for 5 min. The supernatants were collected and assayed for TNF-α.

Mice. Male 6-wk-old inbred C3H/BFeJ mice purchased from Jackson Laboratories (Bar Harbor, ME) were used in all exposure studies. These mice were housed in our institution's rodent vivarium, fed a normal diet (Formulab chow 5008, PMI, Richmond, IN) provided water ad libitum, maintained on wood chip bedding (Northeastern Products, 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 before initiation of this study.

Exposure apparatus and exposure protocol. Exposures were performed in a 20-liter exposure chamber with a Pitt no. 1 nebulizer, with the solutions for inhalation exposure supplied by a syringe pump. In each experiment, groups of 20–24 mice were challenged by inhalation with LPS or saline solution over 4 h. Thirty minutes before inhalation challenge, each mouse was anesthetized (Metofane, Pitman-Moore, Mundelein, IL), intubated with a 24-gauge Jelco intravenous catheter (Johnson and Johnson, Arlington TX), and intratracheally instilled with 50 μl of either a LPS antagonist or PFS. The dose of the LPS antagonist was either 1, 10, or 100 μg and was based on both toxicity studies and the prior experience by Jagielo et al. (18) with RsDPLA. After the inhalation challenge, the animals were euthanized by cervical dislocation, the diaphragm was punctured to deflate the lungs, and whole lung lavage was performed.

ROFA was obtained from the US Environmental Protection Agency, and in these experiments, ROFA was used as a negative control to evaluate the specificity of action of the LPS antagonists. Anesthetized mice were intratracheally instilled with sterile saline (50 μl) or a sonicated solution of ROFA (60 μg of ROFA in 50 μl of saline). This dose is consistent with occupational exposures and has been shown to cause polymorphonuclear neutrophils (PMNs) to be recruited to the airspaces (16). Using the LAL assay, we found that the

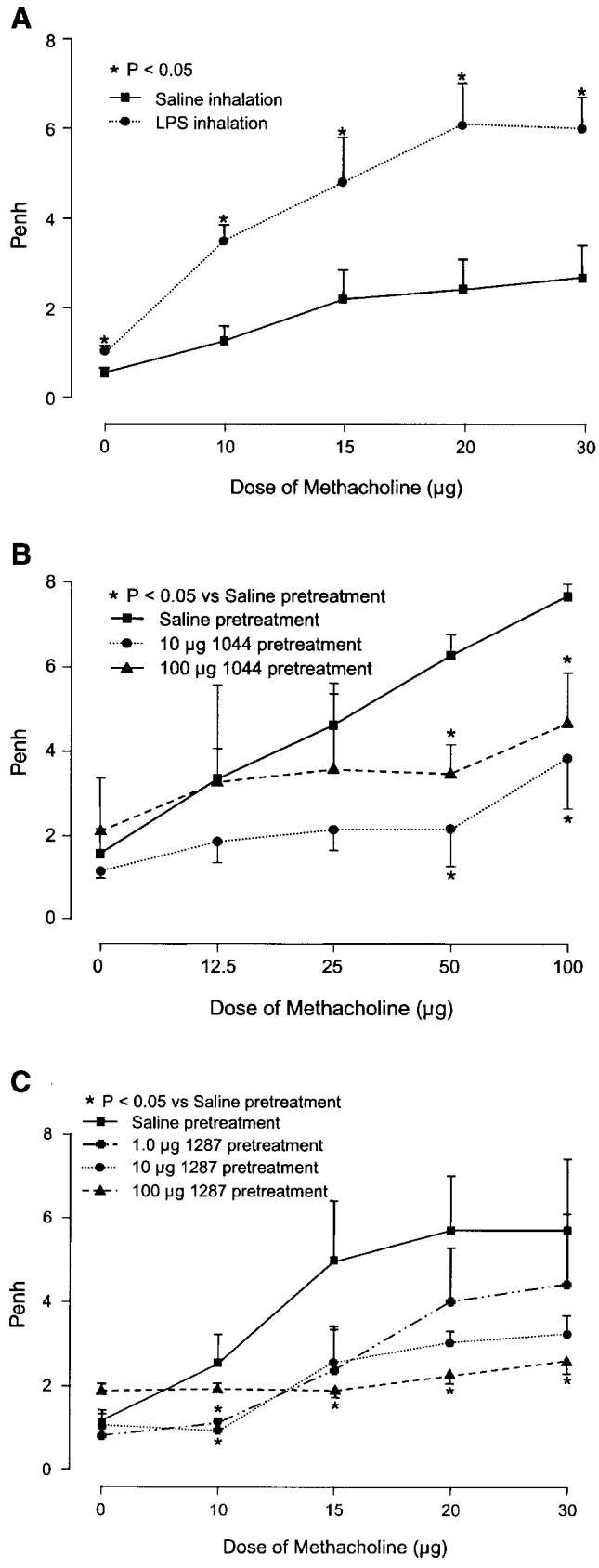

Fig. 3. Enhanced pause (P_{enh}) immediately after inhalation of LPS in C3H/BFeJ mice. P_{enh} was measured after LPS inhalation challenge after increasing doses of inhaled methacholine as described in METHODS. *A*: P_{enh} after inhalation of saline or LPS. In addition, before the LPS inhalation, mice were pretreated with saline or either 1044 (*B*) or 1287 (*C*). Values are means ± SE.

ROFA solution had nondetectable concentrations of endotoxin.

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 and collected; the process was repeated six times. Lung lavage fluid was processed by the standard method of Schwartz et al. (31).

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

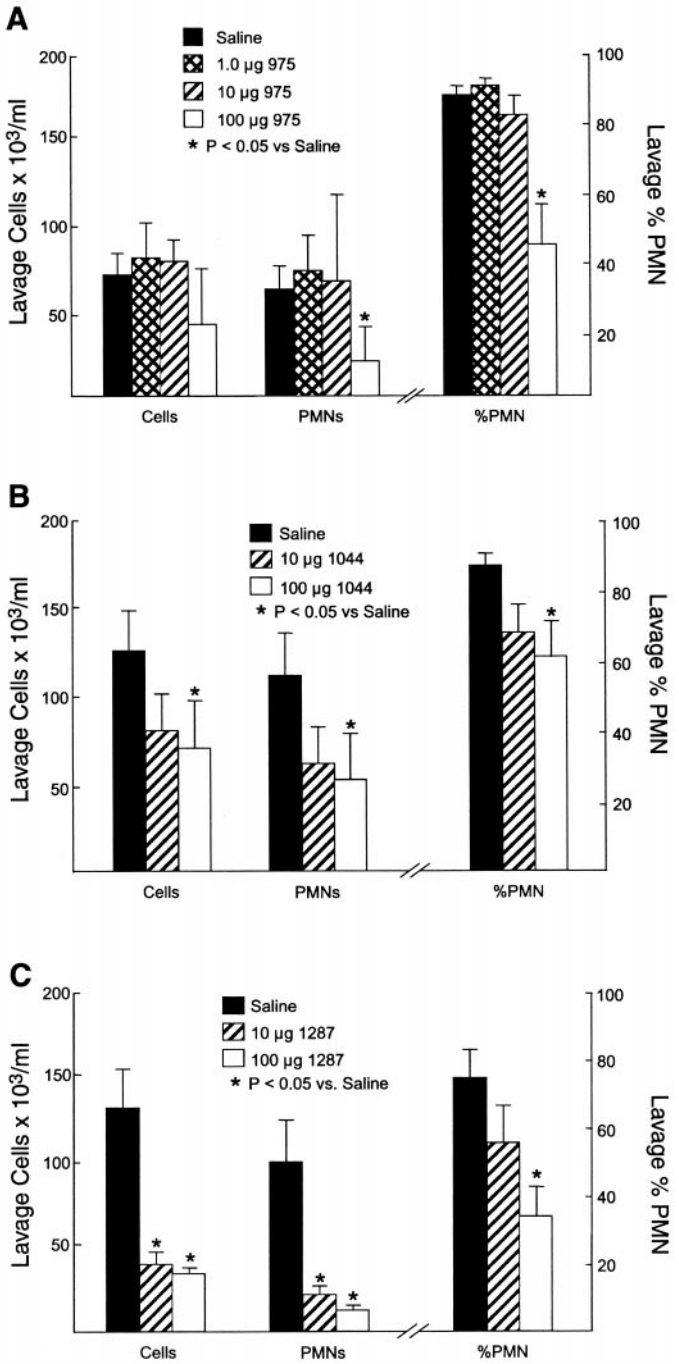

Fig. 4. Lung lavage fluid concentration of total cells and PMNs and percentage of PMNs immediately after inhalation of LPS in C3H/BFeJ mice. Mice were pretreated with saline as a control or with either 975 (*A*), 1044 (*B*), or 1287 (*C*). Values are means ± SE.

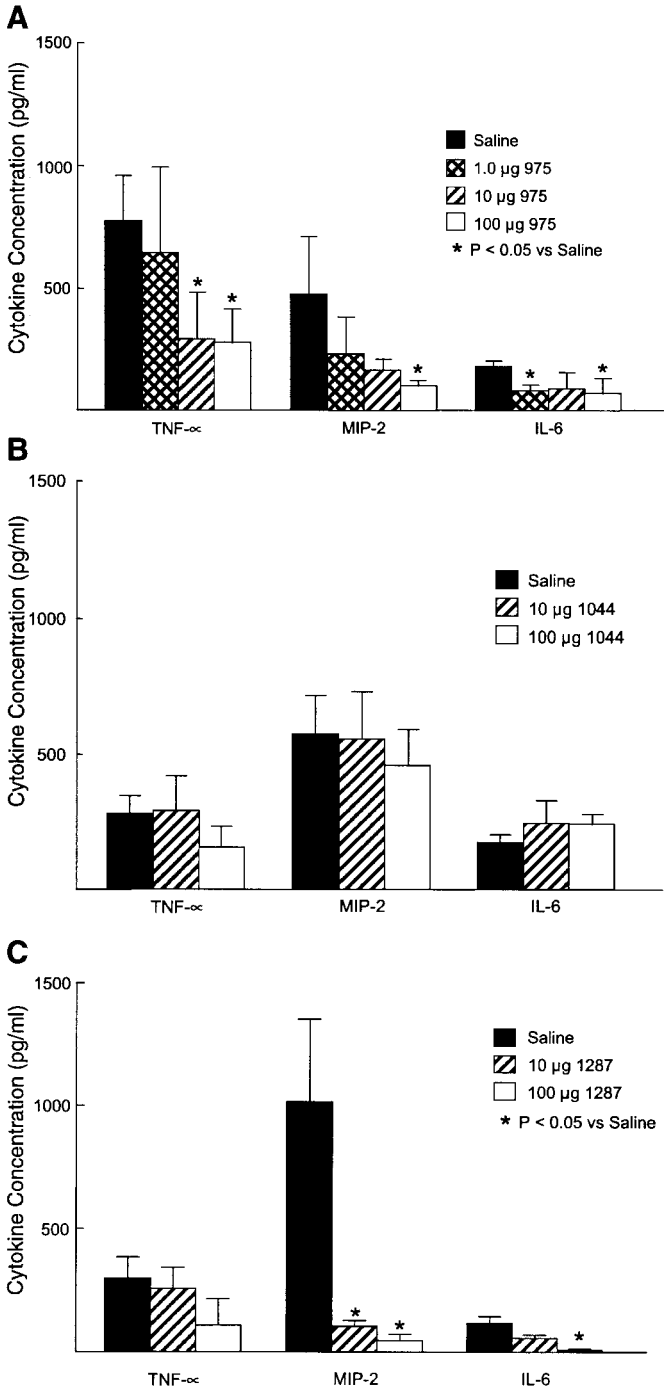

Fig. 5. Lung lavage fluid concentration of TNF-α, macrophage inflammatory protein (MIP)-2, and interleukin (IL)-6 immediately after inhalation of LPS in C3H/BFeJ mice. Mice were pretreated with saline as a control or either 975 (*A*), 1044 (*B*), or 1287 (*C*). Values are means ± SE.

Assessment of pulmonary function. The mice were placed in an 80-ml whole body plethysmograph (Buxco Electronics, Troy, NY) ventilated by bias airflow at 0.2 l/min. This unit is interfaced with differential pressure transducers, analog-to-digital converters, and computers. The breathing patterns and pulmonary function of each individual mouse were monitored over time, and direct measurements were made of respiratory rate, pressure changes within the plethysmograph, and "box flow" (the difference between the animal's nasal airflow and the flow induced by thoracic movement). Box flow varies in the presence of airway obstruction because of pulmonary compression (due to forced expirations). The Buxco system measures both the magnitude of the box pressure variations and the slope of the box pressure; associated software also evaluates the wave shape, which is changed most dramatically during early expiration. Estimates of airway resistance are expressed as enhanced pause (P_{enh}) as given by the equation $P_{enh} = [(T_E/40\% T_r) - 1] \times (P_{ef}/P_{if}) \times 0.67$, where T_E is expiratory time, T_r is relaxation time, P_{ef} is peak expiratory flow, and P_{if} is peak inspiratory flow. P_{enh} has been shown to correlate empirically with airway resistance (33); however, factors other than airway resistance may affect the measurement of P_{enh}.

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 corn dust extract solutions after pretreatment with LPS antagonists and *2*) comparison of the in vivo inflammatory and physiological responses as assessed by both whole lung lavage fluid and airway physiology in mice exposed to LPS after pretreatment with LPS antagonists or sterile saline intratracheally. Statistical comparisons for continuous data were made with the Mann-Whitney *U*-test (15).

RESULTS

The in vitro stimulatory effect of the LPS antagonists (975, 1044, and 1287) on THP-1 cells was determined by performing dose-response experiments in which THP-1 cells were incubated with increasing concentrations of the LPS antagonists (0.01–100 μg/ml) over 24 h. The supernatant fluid was assayed for TNF-α. No TNF-α was detected in the supernatant or cell lysate at any of the concentrations (0.01–100 μg/ml) with any of the LPS antagonists (data not shown). These results differ from the previous findings of Jagielo et al. (18) with RsDPLA where 100 μg/ml of RsDPLA caused substantial release of TNF-α. In contrast, *E. coli* LPS at a concentration of 0.01 μg/ml caused substantial release of TNF-α by THP-1 cells (Fig. 1*A*). To determine whether the LPS antagonists (975, 1044, and 1287) could inhibit the effect of LPS, we incubated THP-1 cells with LPS (endotoxin activity 0.01 μg/ml) and increasing concentrations of the LPS antagonists (0.01–100 μg/ml). Although 0.01 μg/ml of 975 significantly inhibited the release of TNF-α by THP-1 cells, 10 μg/ml of 975 were much more effective and 100 μg/ml of 975 entirely blocked the release of TNF-α by THP-1 cells stimulated with LPS (Fig. 1*B*). Similar results were observed with 1044 and 1287 (Fig. 1, *C* and *D*, respectively).

To determine the effect of the LPS antagonists (975, 1044, and 1287) on airway inflammation, we compared intratracheal instillation of these compounds at doses of 1, 10, and 100 μg to the inflammatory effects of sterile saline. No significant differences were noted in the estimates of airway reactivity (P_{enh}), the concentration of cells, the percentage of PMNs, or the concentration of either TNF-α or MIP-2 in the lavage fluid between the mice instilled with saline and those instilled with any of the LPS antagonists at any of the concentrations tested (Fig. 2). These results also differ

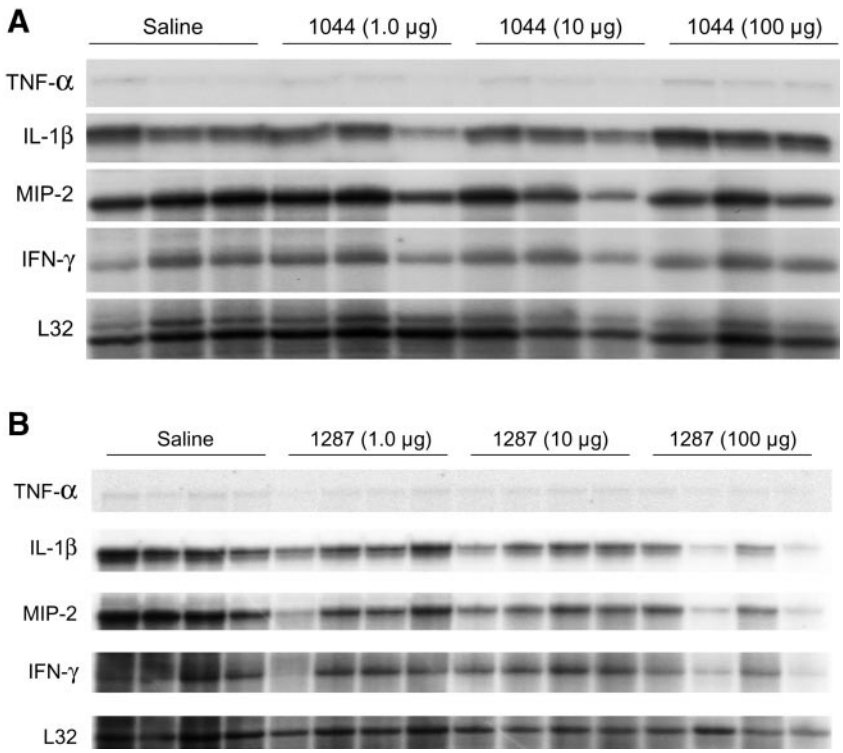

Fig. 6. RNase protection assay of total RNA obtained from lungs immediately after inhalation of LPS in C3H/BFeJ mice. Mice were pretreated with saline as a control or either 1044 (*A*) or 1287 (*B*). Equivalent amounts of RNA were examined in each sample as judged by the amount of L32, which encodes a ubiquitously expressed ribosome subunit protein. IFN-γ, interferon-γ.

from the previous findings of Jagielo et al. (18) with RsDPLA where 100 μg of intratracheally instilled RsDPLA caused substantial PMN recruitment to the lower respiratory tract. These results suggested that we could safely use up to 100 μg of any of the synthetic LPS antagonists to prevent the inflammatory effects of inhaled LPS.

Inhalation of LPS (in comparison to saline) resulted in enhanced airway reactivity to methacholine (Fig. 3*A*). Pretreatment with the LPS antagonists (1044 and 1287; airway physiology not conducted with 975) resulted in less LPS-induced airway hyperreactivity (Fig. 3, *B* and *C*, respectively). Pretreatment with 1044 at 10 or 100 μg intratracheally (compared with pretreatment with saline) resulted in less airway reactivity to methacholine (lower P_{enh} values) after inhalation of LPS (Fig. 3*B*). Similar reductions in LPS-induced airway hyperreactivity were observed after intratracheal treatment with 1287 (Fig. 3*C*).

After intratracheal instillation with one of the LPS antagonists (975, 1044, and 1287) or HBSS, groups of 10 mice underwent inhalation challenge to LPS (airborne levels of endotoxin 3.4–7.2 μg/m^3). Mice pretreated with 100 μg of 975 (Fig. 4*A*), 100 μg of 1044 (Fig. 4*B*), or either 10 or 100 μg of 1287 (Fig. 4*C*) had significantly less cellular inflammation in the lower respiratory tract than mice pretreated with saline. Although significant declines in the lavage fluid concentration of TNF-α, IL-6, and MIP-2 were not observed after treatment with 1044, significant declines were observed when mice were treated with either 975 or 1287 before the LPS inhalation (Fig. 5). Pretreatment with 1044 did not appear to alter the concentration of mRNA cytokine in the whole lung homogenate (Fig. 6*A*); however, 1287 at the higher doses clearly decreased the concentration of mRNA for several cytokines (Fig. 6*B*).

To assess the specificity of action of the LPS antagonists, we pretreated mice with either saline or 100 μg of 1287 and then intratracheally instilled ROFA. Pretreatment with 1287 had no effect on ROFA-induced airway reactivity to inhaled methacholine (Fig. 7*A*) or airway inflammation (Fig. 7*B*), suggesting that the effect of 1287 was specific for LPS and not generalizable to other respiratory toxins such as ROFA that have been shown to induce airway hyperreactivity (16).

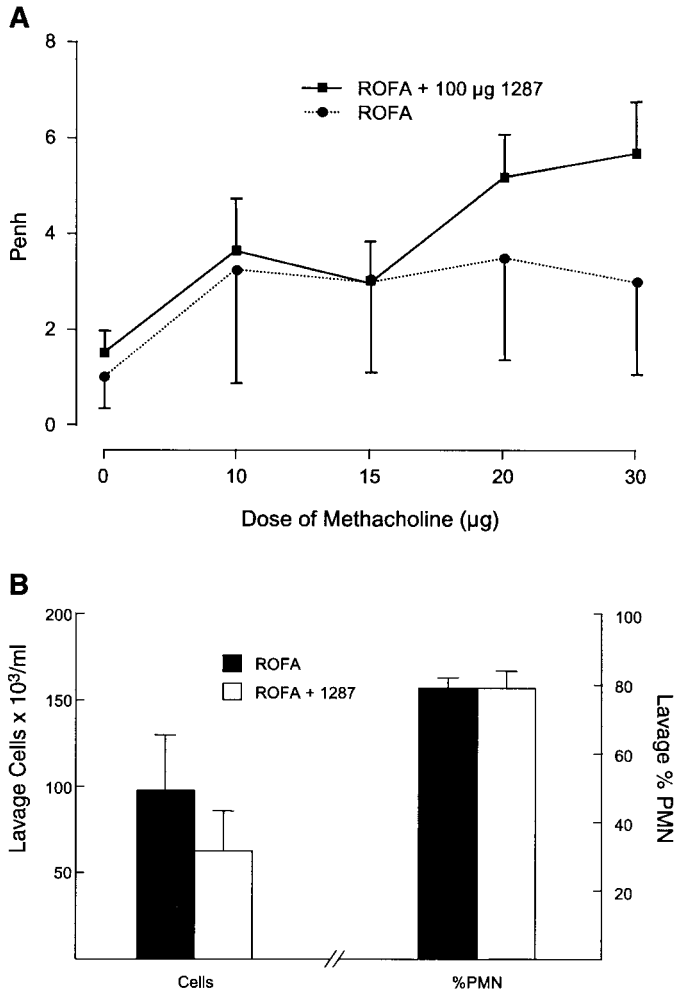

Fig. 7. P_{enh} with increasing doses of methacholine (*A*) and lavage cellularity (*B*) are presented in mice pretreated with either saline or 100 μg of 1287 and then intratracheally instilled with residual oil fly ash (ROFA). Airway reactivity and inflammation of the lower respiratory tract were evaluated 4 h after intratracheal instillation of ROFA.

DISCUSSION

Our study results indicate that pretreatment with synthetic types of LPS antagonists reduced the airway response to inhaled LPS; however, the specific antagonists exhibited different potencies. We have shown that the LPS antagonists *1*) were capable of inhibiting LPS-mediated production and release of TNF-α in vitro by THP-1 cells, *2*) did not induce a discernible inflammatory response in the lung when administered intratracheally at doses of 100 μg, and *3*) substantially reduced the airway hyperreactivity and inflammatory response in the lower respiratory tract caused by inhaled LPS. The LPS antagonist 1287 appears to be more potent than either of the other two antagonists tested. These results suggest that synthetic LPS antagonists may be an effective and potentially safe treatment for endotoxin-induced airway disease as well as for other more common types of asthma.

The results of our study are the first to demonstrate the ability of synthetic LPS antagonists to inhibit endotoxin-mediated lung inflammation with an inhalational model of acute lung inflammation. A previous investigation (35) has 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. Pretreatment of mice with RsDPLA intravenously blocked the rise in serum TNF-α normally observed in mice after an intravenous injection of LPS (26). Furthermore, RsDPLA was protective in preventing endotoxin-mediated death in mice, possibly due to the induction of early endotoxin tolerance (39). A previous investigation by Jagielo et al. (18) with RsDPLA indicated that this relatively inactive

form of LPS reduced the inflammatory response to inhaled LPS. In the current work, we extended these findings and demonstrated that synthetic LPS antagonists are capable of decreasing both the physiological and inflammatory responses to inhaled LPS. The mechanism by which the LPS antagonists act to reduce LPS-induced airway hyperreactivity and lung inflammation is not entirely clear but may be due to direct inhibition of LPS binding to cell receptors in the lung or possibly secondary to the induction of early endotoxin tolerance. The finding that pretreatment with 1287 resulted in inhibition of LPS-induced transcription of key cytokines suggests that the LPS antagonists are functioning at a proximal step in airway inflammation.

Of note, the three synthetic LPS antagonists used in this investigation (975, 1044, and 1287) demonstrated differences in their ability to inhibit LPS-induced airway hyperreactivity and airway inflammation. Although 975 and 1044 did reduce the biological and physiological (only for 1044) responses to inhaled LPS, 1287 was clearly more potent. These differences in efficacy may be attributable to the stability and agonist properties of these agents. For instance, although all of the synthetic LPS antagonists are stable and have demonstrated a potent antagonism of LPS-mediated cellular activation in a variety of in vitro and in vivo assays (7–9), 1287 is much more stable than either 975 or 1044, is very water soluble, has a high antagonist activity profile, and has no discernable agonist activity. Moreover, 1044 can be degraded by loss of a 2′-olefin-bearing side chain, generating some agonistic by-products. Thus it is not surprising that 1287 is more effective in minimizing the physiological and biological responses to inhaled LPS than either of the other LPS antagonists.

Cytokines are involved in the inflammatory response to inhaled endotoxin. In humans and mice, Deetz et al. (12) have previously 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. Because LPS is not directly chemotactic for PMNs (11, 34), endogenous factors must be released to facilitate the rapid movement of PMNs from the vascular space to the airspace after inhalation of LPS. Several lines of investigation suggest that the movement of PMNs from the vascular space to the airspace is a tightly controlled biological process. The process of PMN emigration in the systemic circulation has been fairly well defined and occurs in a series of distinct phases, which actively move the PMNs from the postcapillary venules to the site of inflammation (1, 5, 21). The initial event is rolling or margination, where the PMN is pulled from the stream of circulating cells and loosely tethered to the vascular wall. This is followed by activation and firm adhesion of the PMN to the endothelial cell. A subset of PMNs that become firmly adhered will become flattened and undergo transmigration (1, 13). However, because the diameter of the pulmonary capillary is so much smaller than that of the venule, the leukocyte is already in close contact with the capillary endothelial cells, indicating that the initial phase of rolling is unlikely to be important, and the next phase, firm adhesion, may be quite different from that in the systemic circulation (17). Although the vital elements and kinetics of this process have been fairly well described in the systemic circulation, the transmigration of PMNs from the vascular space to the airspace is unique biologically and structurally (17) and has not been characterized for inhaled stimuli such as LPS. Importantly, results from this study suggest that reducing the concentration of mRNA for IL-1β, MIP-2, and interferon-γ (see Fig. 6*B*) may have a profound effect on the movement of PMNs from the vascular space to the air space after inhalation of LPS.

In conclusion, our results demonstrate that several synthetic LPS antagonists inhibit LPS-induced airway hyperreactivity and lung inflammation, supporting the hypothesis that LPS antagonists may prove effective in treating endotoxin-induced airway disease and other more common forms of asthma. Moreover, intratracheal administration of these agents appears to be nontoxic.

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