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Modified Endotoxin Responses in Rats Pretreated with 1→3-β-Glucan (Zymosan A)

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The present study investigates whether 1→3-β-glucans (zymosan particles) modify the pulmonary response of rats to endotoxin (lipopolysaccharide, LPS). Initial experiments were conducted to establish appropriate doses of LPS and regimens for exposure to zymosan and LPS. Interaction between zymosan and LPS exposures was determined to be the deviation from the sum of the individual effects of these agents. Treatment with zymosan on Day 1 and LPS on Day 2 modified several indices of pulmonary responsiveness, including tumor necrosis factor-α, albumin, and lactate dehydrogenase activity (LDH) in first acellular lavage fluid as well as the levels of chemiluminescence (CL), NO-dependent CL, and nitric oxide production in cultured lavaged alveolar macrophage cells determined 1 day after exposure. No significant deviation from additivity was found for breathing rate increase and polymorphonuclear leukocytes infiltration. Simultaneous administration of zymosan and LPS or administration of LPS before zymosan did not change these indices of pulmonary responsiveness. These data suggest that the inhibitory effect of 1→3-β-glucans on pulmonary responsiveness to endotoxin exposure was apparent only when rats were pretreated with 1→3-β-glucan. These results suggest that complex interaction of components may exist in exposure to organic dusts. Therefore, hazard may not be defined by measuring endotoxin or 1→3-β-glucans alone.

Key Words: **1→3-β-glucans; zymosan; endotoxin; pulmonary inflammation.**

Mold, fungi, and bacteria are important factors in identifying problems associated with indoor air quality. Recently, 1→3-β-glucans, derived from the inner cell wall of yeasts and fungi (DiCarlo and Fiore, 1957), have been suggested to play a role in organic dust toxic syndrome (Fogelmark *et al.,* 1994; Rylander, 1997; Wan and Li, 1999). Exposure to 1→3-β-glucans has been associated with airway inflammation in several occupations, including house waste collection (Thorn *et al.,* 1998), composting (Douwes *et al.,* 2000), sewage treatment (Rylander, 1999a) and paper mills (Rylander *et al.,* 1999b). These symptoms are often referred to as organic dust toxic syndrome (ODTS), which includes fever, chills, and malaise after exposure (Rylander, 1994). Studies have shown that 1→3-β-glucans by themselves can cause an acute inflammatory response in rats (Robinson *et al.,* 1996), which suggests that 1→3-β-glucans may be an important occupational/environmental cause of pulmonary disease. 1→3-β-Glucans are widely distributed in the environment. 1→3-β-Glucans can be found in bird droppings (Rylander *et al.,* 1994), pollen (Rylander *et al.,* 1999a), rowhouses (Thorn and Rylander, 1998), and in patients with pulmonary aspergilloma (Yuasa and Goto, 1997). Measurement of 1→3-β-glucans has been considered as a marker of fungal biomass. Rylander (1999b) suggested that biomass rather than viability correlated more strongly with the symptoms associated with indoor mold exposure. Therefore, measurement of 1→3-β-glucans is one of the important indicators in investigating indoor air quality and problems of occupational or environmental health. Considering the potency and wide distribution of 1→3-β-glucans, there is clearly a need to understand the health effects of 1→3-β-glucans.

Besides their inflammatory property, 1→3-β-glucans also are Biological Response Modifiers (Augustin 1998; Bohn and BeMiller, 1995; Di Luzio, 1983, 1985). This classification was given pharmacologically for immunotherapeutic purposes to those compounds that can stimulate immunity and increase resistance to microbial disease. 1→3-β-Glucans have been shown to enhance the immune system systemically. Therefore, animals treated with 1→3-β-glucans exhibit an enhanced ability to resist microbial invasion, such as bacterial infection. Since 1→3-β-glucans possess these two distinct effects on hosts, it is of interest to investigate how hosts would respond to LPS in the presence of 1→3-β-glucans; i.e., will 1→3-β-glucans have a inhibitory or potentiating effect.

Endotoxin, derived from gram-negative bacteria, is commonly found in organic dust. Endotoxin is a well-known potent pulmonary inflammatory stimulant. 1→3-β-Glucans have been shown to interact with endotoxin and to modify the host response to endotoxin. However, conflicting results suggesting either enhancement (Bower *et al.,* 1986; Cook *et al.,* 1980) or

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inhibitory (Soltys and Quinn, 1999; Vereschagin *et al.,* 1998) effects on host response to endotoxin have been reported. Such differences could depend on experimental conditions. In some studies, 1→3-β-glucans have been suggested to enhance the toxicity of endotoxin. Cook *et al.* (1980) pretreated rats with particulate glucans and reported marked increases in their sensitivity to endotoxic shock. Bower *et al.* (1986) reported that rats pretreated with particulate glucans, but not soluble glucans, became sensitized to endotoxins. Recently, inhibitory effects of 1→3-β-glucans have been reported. Soltys and Quinn (1999) found that *in vivo* treatment of mice with soluble glucans resulted in suppression of proinflammatory cytokines, such as IL-6 and TNF-α production, in response to endotoxin. Vereschagin *et al.* (1998) found that pretreatment of mice with soluble carboxymethyl-β-1,3-glucan (CMG) protected against endotoxin shock. A trend was observed that most of the reported inhibitory effects of 1→3-β-glucans on endotoxin were seen with soluble 1→3-β-glucans, while most of the studies reporting enhancement of endotoxin toxicity used particulate 1→3-β-glucans. This laboratory is interested in inhalation toxicity of particulate 1→3-β-glucans. The particulate form of 1→3-β-glucans is the form most likely found in environmental settings. The particulate type of 1→3-β-glucans has been shown to cause more toxicity than soluble forms (Williams *et al.,* 1991). In a previous study (Young *et al.,* 2001), we demonstrated that intratracheal instillation (IT) of a particulate 1→3-β-glucan (zymosan A) caused a dose-dependent inflammatory response in rats. The present study investigates whether particulate 1→3-β-glucans can modify the pulmonary response of rats to endotoxin.

METHODS

Preparation of Zymosan A and LPS

Zymosan A, from Baker's yeast, was obtained from Sigma Chemical Company (St. Louis, MO). The majority of particles were between 2 and 5 μm in diameter, which is in the range of respirable particles. The particle size distribution in suspension was similar to that previously reported in inhalation studies (Robinson *et al.,* 1996). Zymosan was freshly prepared as an aqueous suspension each time before use.

LPS was purchased from Difco Laboratories (*E. coli* 026:B6, Detroit, MI). LPS was suspended in sterile PBS at the indicated concentration and was freshly prepared before use.

Animals

Specific pathogen-free male Sprague–Dawley rats (~250 g) were purchased from Hilltop Labs (Scottsdale, PA). Rats were kept in cages upon arrival and housed in an AAALAC-approved facility maintained at 23 ± 1°C with 50% relative humidity and a 12-h light/dark cycle for at least 7 days before use. Food and water were given *ad libitum.* Before intratracheal instillation, rats were lightly anesthetized with an ip injection of 0.6 ml 1% (w/v) sodium methohexital (Eli Lilly Co., Indianapolis, IN). The test suspension was quickly instilled into the trachea. Rats received a single dose of the indicated amount (in mg/kg body wt) of zymosan A or LPS. Control animals received the sterile PBS vehicle only. The instillation volume was approximately 0.26 ml per rat.

Dosages Choices for Zymosan and LPS

Dose–response curve in LPS-IT rats. In order to choose a submaximum dose for LPS-IT, separate experiments were conducted to determine the pulmonary response of rats to various doses of LPS (0.1–5 mg/kg body wt). Rats received a single dose of the indicated amount (in mg/kg body wt) of LPS. Control animals received the sterile PBS vehicle only. Rats were euthanized at 1 day post-IT.

Choice of a submaximum dose for zymosan and LPS. A submaximal dose of zymosan was chosen from our previous study (Young *et al.,* 2001). The submaximal dosage of LPS was chosen following the dose–response of the LPS–IT experiment (described above). The doses that were submaximal, but caused a significant level of pulmonary response above control were the following: zymosan 2.5 mg/kg of body wt; LPS 0.1 mg/kg of body wt.

Combined Treated Groups and Calculation of Expected Values

Three experimental groups were investigated to evaluate the interaction of 1→3-β-glucans and endotoxin: Group 1, IT zymosan at day 1 and IT LPS at day 2; Group 2, IT LPS at day 1 and IT zymosan at day 2; Group 3, IT zymosan and LPS at day 1, IT PBS at day 2. Four additional groups were added as controls of zymosan or LPS alone at different days: Group 4, IT zymosan at day 1, IT PBS at day 2; Group 5, IT LPS at day 1, IT PBS at day 2; Group 6, IT PBS at day 1, IT zymosan at day 2; Group 7, IT PBS at day 1, IT LPS at day 2; Group 8, PBS-treated control. At day 3, the following pulmonary responses were monitored: (1) breathing frequency, (2) differential cell counts of bronchoalveolar lavage (BAL) cells, (3) chemiluminescence (CL), and (4) NO-dependent CL as a measure of alveolar macrophage activation, (5) nitric oxide production from alveolar macrophages, and (6) tumor necrosis factor-α (TNF-α) levels, (7) albumin levels, and (8) lactate dehydrogenase (LDH) activity in the first acellular lavage fluid. Interaction between zymosan and endotoxin exposures was determined as the deviation from the sum of the individual effects of these agents.

The expected values were calculated using Equation (1). The results from the combined zymosan and LPS treatment groups were compared to the expected value with their matched control groups. For example, the results from group 1 (1st day zymosan, 2nd day LPS) would be compared to the results from 1→3-β-glucans-IT at day 1 (group 4) plus results from LPS-IT at day 2 (group 7) minus the result from PBS-IT group (group 8).

$$\text{Expected value} = (1\rightarrow 3\text{-}\beta\text{-glucans-IT at day } x) + (\text{endotoxin-IT at day } y) - (\text{PBS-IT}) \quad (1)$$

Monitors of Pulmonary Responsiveness

Breathing rate measurement. Breathing frequencies were determined using a flow plethysmograph that has been previously described in detail (Frazer *et al.,* 1997). The plethysmograph chamber was constructed of an acrylic tube enclosed at both ends. One end of the chamber had a circular port that contained four 400-mesh stainless steel screens. Pressure variations across the screens generated by flow into and out of the chamber were measured with a pressure transducer (Setra, Inc., Foxborough, MA). A digital oscilloscope (Tektronix, Inc., Wilsonville, OR) was used to record flow signals, which were transferred to a digital computer for analysis. To measure the rat breathing frequencies, a rat was placed in the flow plethysmograph, equilibrated with 10% CO_2, and its average breathing frequency was calculated based on the time between zero crossings of the flow signal at the beginning and end of an inhalation–exhalation cycle.

Bronchoalveolar lavage and biochemical assay of BAL fluid (BALF). Rats were anesthetized with 0.7 ml of sodium pentobarbital (50 mg/kg ip; Butler, Columbus, OH). The trachea was cannulated and the lungs were lavaged with ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered solution (PBS: 145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM glucose; pH 7.4) using a volume of 6 ml for the first lavage and 8 ml for

TABLE 1
Dose Response to LPS Exposure[a]

LPS (mg/kg)	Albumin (mg/ml)	LDH (U/L)	Chemiluminescence[b]	NO-dependent chemiluminescence[b]	PMN ($\times 10^{-6}$ cells)
Control	0.15 ± 0.02	43.0 ± 3.9	2.57 ± 0.45	0.80 ± 0.16	1.8 ± 0.5
0.1	0.21 ± 0.01*	79.5 ± 3.4*	83.8 ± 28.4*	47.8 ± 14.1*	8.7 ± 1.7*
0.5	0.27 ± 0.02*	162.5 ± 15.6*	135.4 ± 14.5*	71.8 ± 6.0*	21.6 ± 1.9*
1	0.24 ± 0.06*	147.8 ± 17.4*	89.8 ± 14.3*	26.1 ± 5.4*	23.6 ± 3.1*
2.5	0.22 ± 0.02*	197.5 ± 32.9*	94.2 ± 9.2*	27.4 ± 12.0*	22.1 ± 5.3*
5	0.21 ± 0.03*	111.0 ± 23.6*	119.4 ± 32.7*	27.5 ± 18.4*	38.2 ± 7.0*

[a] Values expressed as means ± SEM of four rats.
[b] Zymosan-stimulated chemiluminescence (cpm $\times 10^{-5}$/0.25 $\times 10^{6}$ AM/15 min).
* Significantly higher than control level.

subsequent lavages. A total of 80 ml of BALF was collected from each rat and centrifuged at 1700 rpm for 10 min at 4°C. The supernatant from the first lavage was saved for analysis of TNF-α, albumin, and LDH. Supernatants from other lavages were decanted and discarded. All cell pellets from an individual rat were combined as the BAL cells, resuspended in 1 ml Hepes-buffered solution (145 mM NaCl, 5 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 1 mM $CaCl_2$, and 5.5 mM D-glucose; pH 7.4), and placed on ice. Aliquots of cell suspensions from each rat were taken for the determination of the total cell and differential cell counts using a Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL). The remaining BAL cells were used to determine alveolar macrophage (AM) chemiluminescence and secretion of nitric oxide.

Albumin content of the acellular BAL fluid from the first lavage was used to evaluate damage to the alveolar–capillary barrier. The activity of LDH, a cytosolic enzyme, in the first acellular BAL fluid was assayed to evaluate general cellular damage. The albumin content and LDH activity were measured using an automated Cobas Fara II Analyzer (Roche Diagnostic Systems, Montclair, NJ). The albumin content was determined colorimetrically at 628 nm based on albumin binding to bromocresol green (albumin BCG diagnostic kit, Sigma Chemical Company) and expressed as mg/ml BAL fluid. LDH activity was measured by the formation of NADH and expressed as U/L BALF using Roche Diagnostic reagents and procedures (Roche Diagnostic Systems).

Alveolar macrophage chemiluminescence. The alveolar macrophage chemiluminescence assay was conducted in a 0.25-ml reaction mixture of Hepes-buffered solution. Resting AM CL was determined by incubating 0.25×10^6 AM at 37°C for 20 min and then adding 0.008 mg% (w/v) luminol (Sigma Chemical Company) followed by the measurement of CL for 15 min. To determine zymosan-stimulated AM CL, the reaction mixture was modified to include 0.5 mg of nonopsonized zymosan (Sigma Chemical Company), which was added immediately prior to measurement of CL. Measurement of AM CL was conducted with an automated luminometer (Berthold Autolumat LB 953, Wallace, Inc., Gaithersburg, MD) at 390–620 nm for 15 min, and the integral of counts per minute (cpm) verses time was calculated. Zymosan-stimulated CL was calculated as the cpm in the zymosan-stimulated assay minus the cpm in the resting assay. The NO-dependent CL was determined by adding 1 mM *N*-nitro-L-arginine methyl ester HCl (L-NAME, Sigma Chemical Company) to the cells prior to preincubation. NO-dependent CL was calculated as the difference between zymosan-stimulated CL measurements in the absence and presence of L-NAME (an inhibitor of nitric oxide synthase).

Primary BAL Cell Culture

The BAL cells from each rat were plated in RPMI-1640 culture medium (RPMI, Sigma Chemical Co.) containing 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, and 10% heat-inactivated fetal bovine serum. Aliquots of cell suspensions from each rat, adjusted to 0.5×10^6 AM, were added to each well of a 24-well tissue culture plate. Macrophages were allowed to adhere to the plastic culture plate for 120 min in a humidified incubator. The nonadherent BAL cells were then removed by rinsing the monolayers three times with RPMI media. The AM-enriched adherent cells were incubated (37°C and 5% CO_2) in fresh RPMI medium for 24 h. The AM-conditioned media were collected and the supernatant was analyzed for nitric oxide or stored at −70°C for later analysis for nitric oxide.

Determination of TNF-α and Nitric Oxide

First lavage acellular fluid was collected and TNF-α was measured using a TNF-α ELISA kit from Biosource International (Camarillo, CA) according to the manufacturer's protocol. Nitric oxide in AM-conditioned media was determined as nitrite (NO_2^-) with Griess reagent as described by Green *et al.*, (1982). Nitrite concentrations were determined using a standard curve prepared from sodium nitrite with a linear range from 1.6 to 206 μM. Three replicates were performed for each sample in an experiment.

Effect of in Vivo Zymosan Exposure on ex Vivo LPS-Induced Nitric Oxide Production by AM

Ex vivo LPS-induced NO production was assessed as a measurement of the ability of macrophage to respond to bacterial (LPS) invasion. The effect of pretreatment with zymosan *in vivo* on nitric oxide production was studied by culturing the BAL cells from group 6 (1st day IT with PBS, 2nd day IT with zymosan). These BAL cells were cultured for 120 min and then were rinsed twice to obtain AM-enriched adherent cells. These AM-enriched cells were then treated with 10 μg/ml LPS for an additional 24 h. The NO production was measured by the Griess reaction.

Statistical Analysis

All data were expressed as the mean ± SEM. Statistical analysis was performed using SigmaStat version 2.0. software (Jandel Corporation). For multiple comparisons (e.g., the determination of a optimum LPS dose), the one-way ANOVA for comparing several treatment groups with one control was used. Significance was set at $p \leq 0.05$. For evaluating interaction among zymosan and LPS treatments, two-way ANOVA with Tukey test was used for all pairwise multiple comparison procedures.

RESULTS

Dose Response to LPS Exposure

Table 1 shows the dose response to LPS exposure. The following pulmonary indicators were measured: (1) breathing

TABLE 2
Pulmonary Response after Zymosan and/or LPS Exposure[a]

	PBS control (day 1, PBS/day 2, PBS/ day 3, euthanized)	Zymosan (day 1, zymosan/day 2, PBS/ day 3, euthanized)	LPS (day 1, PBS/day 2, LPS/day 3, euthanized)	Zym + LPS (day 1, zymosan/day 2, LPS/ day 3, euthanized)	Expected additive value (zymosan + LPS − PBS)
PMN ($\times 10^6$ cells)	2.7 ± 0.3	17.7 ± 2.4	15.2 ± 3.9	25.2 ± 4.9	30.8 ± 4.3
Net breathing frequency increase (breaths/min)	0.59 ± 0.59	61.2 ± 10.3	4.9 ± 3.5	89.9 ± 9.4	65.5 ± 14.4
Albumin (mg/mL)	0.14 ± 0.01	0.27 ± 0.02	0.16 ± 0.01	0.21 ± 0.02*	0.30 ± 0.02
LDH (U/L)	48.8 ± 1.7	145.5 ± 8.2	102.7 ± 18.1	135.5 ± 17.2*	207.5 ± 17.3
Particle stimulate-chemiluminescence (cpm $\times 10^{-5}/0.25 \times 10^6$ AM/15 min)	2.4 ± 0.6	13.3 ± 3.8	74.8 ± 11.2	46.1 ± 10.1*	85.8 ± 11.2
NO-dependent chemiluminescence (cpm $\times 10^{-5}/0.25 \times 10^6$ AM/15 min)	0.9 ± 0.2	4.5 ± 1.9	47.8 ± 9.2	11.2 ± 5.7*	51.4 ± 8.8

[a] Values expressed as means ± SEM of four rats.
* Significantly lower than the expected additive value.

frequency, (2) differential cell counts of bronchoalveolar lavage cells, (3) alveolar macrophage chemiluminescence and (4) NO-dependent chemiluminescence as a measure of activation, and (5) albumin level and (6) lactate dehydrogenase activity in the first acellular lavage fluid.

At all LPS concentrations tested the indices measured were significantly higher in general than the control (Table 1). Exposure to LPS caused an increased BALF albumin level. However, no statistical difference was found among different LPS concentrations. LPS exposure also significantly increased the LDH activity of the BALF at all LPS concentrations tested. Except for 1 and 5 mg/kg of LPS, the LDH activity tended to increase with increasing LPS dose. This suggests that LPS exposure caused cytotoxicity. From the differential cell counts of bronchoalveolar lavage cells, a dose-dependent polymorphonuclear leukocytes (PMN) infiltration in response to LPS ($r^2 = 0.77$, linear regression) was observed. Breathing frequency showed a similar relationship with LPS exposure, with increasing breathing rate showing a correlation coefficient of 0.86 with LPS dose (data not shown). These two indicators suggest that the inflammatory response correlated well with LPS dose. The chemiluminescence increased by more than 30-fold after exposure to 0.1 mg/kg of LPS. Statistically significant elevations in the generation of reactive oxygen species by AM were observed at all LPS concentrations tested. NO-dependent chemiluminescence showed a similar response as total chemiluminescence, with all concentrations of LPS causing a statistically significant increase above control levels. Both total and NO-dependent chemiluminescence seemed to peak at 0.5 mg/kg of LPS.

Choice of IT Dose for LPS and Zymosan A

A LPS dose of 0.1 mg/kg was chosen for the subsequent combination studies based on the following considerations: (1) 0.1 mg/kg showed a statistically greater response than control for all the parameters examined. (2) For chemiluminescence, no further increase of response was observed for LPS concentrations above 0.5 mg/kg. (3) High cytotoxicity (indicated as BALF LDH activity greater than 100 U/L) was observed from 0.5 to 2.5 mg/kg of LPS. Therefore, a dose of 0.1 mg/kg appeared to be a submaximum dose for LPS, causing inflammation without excessive cytotoxicity.

For zymosan, the dose chosen was 2.5 mg/kg body wt. This was based on our previous study of the dose dependence of inflammatory responses in rats induced by intratracheal instillation of zymosan (Young *et al.*, 2001). This dose was a submaximum dose, since the pulmonary response to 5 mg/kg was greater than that for 2.5 mg/kg of zymosan. For all the parameters examined, the response to 2.5 mg/kg of zymosan was statistically higher than control.

Regimen Determination Following Combined Exposure to Zymosan and LPS

The combined exposure to zymosan and LPS was evaluated after both simultaneous and sequential exposure. However, after analysis, only group 1 (1st day zymosan, 2nd day LPS) resulted in pulmonary response levels that were statistically different from the sum of the separate exposures to zymosan or LPS. Therefore, these results focus on the pulmonary response of group 1 only (Table 2).

Pulmonary Responsiveness Endpoints

Breathing frequency. Instillation of dust particles has been shown to cause a change in respiratory rate (Porter *et al.*, 1999; Robinson *et al.*, 1997). Breathing frequencies were determined immediately prior to the IT administration of zymosan and immediately prior to euthanasia at day 3. In a previous exper-

iment, when rats were exposed to various doses of zymosan (Young *et al.*, 2001), a direct correlation between net breathing frequency increase and dosage of zymosan was observed (correlation coefficient, $r^2 = 0.95$). The net breathing frequency increase was referred to here as the breathing frequency difference between day 3 and pre-IT breathing frequency. Instillation of zymosan or LPS induce a significant increase in breathing frequency (Table 2). The increase in breathing frequency in animals treated with zymosan on day 1 followed by LPS on day 2 was not significantly different from the expected additive value (the interaction between zymosan and LPS was not significant by two-way ANOVA, $p = 0.115$).

Cell differentials. Bronchoalveolar lavage was used to assess the effect of zymosan or LPS on cellular differentials. Inflammatory response was monitored as neutrophil infiltration, i.e., the number of polymorphonuclear leukocytes harvested by BAL. Table 2 shows that, in the control rat group, a low number of PMN in BALF was observed. Treatment with zymosan-IT or LPS-IT alone induced PMN infiltration into lung alveoli. In the group where zymosan then LPS was treated (Group 1, 1st day zymosan, 2nd day LPS), PMN yield was higher than either zymosan or LPS alone. This PMN infiltration was not significantly different than the expected additive value (the interaction between zymosan and LPS was not significant by two-way ANOVA, $p = 0.431$).

Albumin concentration in the first acellular BALF. Albumin concentration was measured in the first acellular lavage fluid. As expected, albumin levels were low in BALF from the PBS control rats (Table 2). BALF albumin increased after exposure to zymosan but not LPS alone. For the group where zymosan then LPS were administered (Group 1, 1st day zymosan, 2nd day LPS) albumin was higher than PBS control. However, damage to the alveolar air–blood barrier was significantly lower than the expected additive value for the response to zymosan and LPS alone (the interaction between zymosan and LPS was significant by two-way ANOVA, $p = 0.021$).

LDH activity in the first acellular BALF. The cytotoxicity of exposure was evaluated by measuring the LDH activity in BALF. Exposure to either zymosan or LPS alone increase LDH activity (Table 2). However, in the zymosan then LPS group (Group 1, 1st day zymosan, 2nd day LPS) BALF LDH activity was significantly lower than the expected additive value of the separate exposures ($p = 0.018$).

Alveolar macrophage chemiluminescence. Chemiluminescence assay was conducted to assess the potential inflammatory activation of alveolar macrophages. Exposure to either zymosan or LPS increased AM CL above the PBS control level (Table 2). However, in the zymosan then LPS group (Group 1, 1st day zymosan, 2nd day LPS), CL was significantly lower than the expected additive value of the separate exposures ($p = 0.026$).

Alveolar macrophage NO-dependent chemiluminescence. The NO-dependent CL assay was conducted to assess NO production by the AM. The NO-dependent CL was estimated by adding 1 mM L-NAME to the samples and measuring the difference between zymosan-stimulated CL in the absence and presence of this NOS inhibitor. Exposure to either zymosan or LPS increased NO-dependent CL above control (Table 2). However, in the zymosan then LPS group (Group 1, 1st day zymosan, 2nd day LPS) NO-dependent CL was significantly lower than the expected additive value of the separate exposures ($p = 0.004$).

TNF-α production in the first acellular BALF. TNF-α production was measured as the cytokine present in the first acellular BALF collected from rats in the various exposure groups. Figure 1 shows that zymosan or LPS exposure increased pulmonary TNF-α production. However, the TNF-α levels for the group where zymosan then LPS was treated (Group 1, 1st day zymosan, 2nd day LPS) were significantly lower than the expected additive value of the separate exposures ($p = 0.049$).

Effect of in vivo zymosan exposure on ex vivo LPS-induced nitric oxide production from AM. Nitric oxide is one of the cellular messages released by AM after LPS or zymosan stimulation. No detectable NO was produced by AM from PBS-IT control rats (Fig. 2). However, when these AM were exposed to LPS *ex vivo* for 24 h, significant NO production (41 μM) was observed. Exposure to zymosan *in vivo* induced nitric oxide production (161.8 μM) by AM cells. However, *in vivo* zymosan treatment followed by *ex vivo* LPS stimulation resulted in significantly lower nitric oxide production than the expected additive value ($p = 0.05$).

DISCUSSION

The interaction between 1→3-β-glucans and endotoxin has been a topic of interest ever since 1→3-β-glucans were first identified in occupational/environmental exposures. 1→3-β-Glucans have shown to be beneficial due to their immunomodulating activity (Di Luzio 1985). However, whether inhaled 1→3-β-glucans affect pulmonary inflammation induced by inhalation of organic dust is still unclear. Inhibition of cytokines production is a possible mechanism to reduce inflammation. 1→3-β-Glucans have been reported to reduce TNF-α production in response to bacterial LPS *in vitro* (Hoffman *et al.*, 1993) and *in vivo* (Soltys and Quinn, 1999). However, the biological effects of 1→3-β-glucans are partially dependent on the form tested (particulate versus soluble) as well as the route of exposure. Early studies on 1→3-β-glucans have focused on pharmaceutical usage, in which case, the soluble form of 1→3-β-glucans is the form of choice. On the other hand, particulate 1→3-β-glucans are the likely forms found in the environment. Particulate forms of 1→3-β-glucans have been shown to cause more toxicity than soluble 1→3-β-

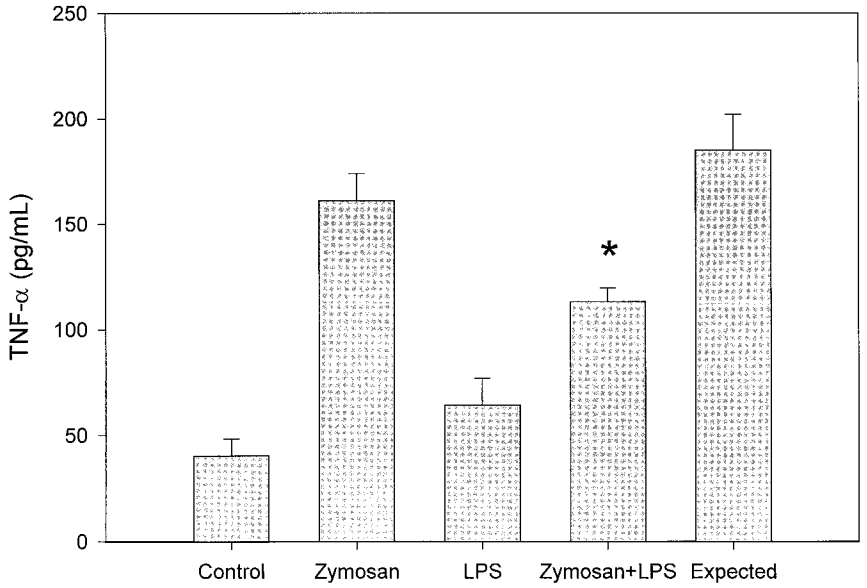

FIG. 1. Inhibition of responsiveness to LPS by zymosan pretreatment. TNF-α levels were measured in the first acellular BALF. Euthanasia was on day 3 after administration of PBS or zymosan on day 1 or PBS or LPS on day 2. The expected value was calculated from the expected additive value, i.e., expected = zymosan + LPS − PBS. *Significantly lower stimulation than the expected additive effect from zymosan or LPS exposure alone ($p \leq 0.05$). Values are means ± SEM of five rats.

glucans (Williams *et al.*, 1991). In order to have a better understanding of the pulmonary response to the inhalation of particulate 1→3-β-glucans found in organic dusts, the present study focused on the particulate form only.

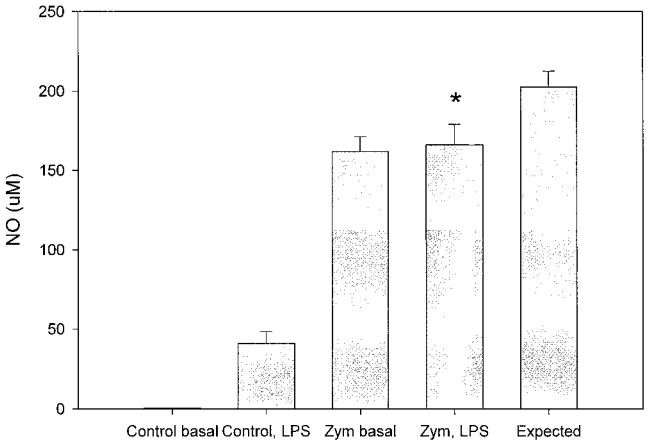

FIG. 2. *In vivo* zymosan exposure decreased nitric oxide production from AM in response to *ex vivo* LPS exposure. AM (0.5×10^6 cells/well) were plated in a 24-well plate and cultured for 24 h before measurement of NO levels in the culture medium. The control basal was unstimulated cultured AM cells harvested from PBS-IT control rats. The control LPS was cultured LPS-stimulated (10 μg/ml LPS) AM cells harvested from PBS-IT control rats. The zymosan basal was cultured unstimulated AM cells harvested from day 1 PBS-IT, day 2 zymosan-IT rats. The zymosan LPS was LPS-stimulated cultured AM cells from day 1 PBS-IT, day 2 zymosan-IT rats. The expected value was calculated from the expected additive value, i.e., expected = zymosan basal + control LPS − control basal. *Significantly lower than expected additive effect ($p \leq 0.05$). Values are means ± SEM of 5 to 10 rats.

Breathing frequency increase and PMN infiltration post-IT are both sensitive indicators of pulmonary inflammation. The results of the present study suggest that zymosan did not inhibit this inflammatory reaction to subsequent LPS exposure; that is, increases in breathing rate and PMN infiltration in response to zymosan followed by LPS were no different from that expected from the additive effects of separate exposure to zymosan A and LPS. Therefore, the presence of 1→3-β-glucans did not modify the inflammatory response to LPS. Zymosan was used as a crude preparation of 1→3-β-glucans in this experiment. The term "zymosan" refers to crude yeast cell wall preparations consisting chiefly of protein–carbohydrate complexes. Riggi and Di Luzio (1961) reported that the active fraction of zymosan was 1→3-β-glucans, which induced proliferation and activation of the reticuloendothelial system. The killing of infecting organisms was not manifested by zymosan itself but by the interaction with other components of the host–defense system such as complement (Pillemer and Ecker, 1941) and properdin (Pillemer and Ross, 1955). Several 1→3-β-glucans have been found to have antiinflammatory ability. This may be related to their role in inhibiting the alternative pathway of complement by binding porperdin (Stone and Clarke, 1992). However, in the present study, LPS-induced elevation of breathing frequency and PMN infiltration were not inhibited by prior exposure to zymosan. This discrepancy may due to the form of 1→3-β-glucan used (soluble versus particulate) and/or the route of exposure (IV vs IT).

Although LPS-induced inflammation was not decreased, an inhibition of LPS-induced cytotoxicity (LDH activity and albumin level in first acellular BALF), macrophage activation

(chemiluminescence), and cytokine (TNF-α) production were observed in the rats pretreated with zymosan. This inhibition was not observed when rats were treated with LPS before zymosan exposure or when rats were exposed to LPS and zymosan simultaneously. Inhibition of LPS-induced macrophage activation (chemiluminescence) by pretreatment with zymosan was also reported in a guinea pig inhalation model (Robinson *et al.,* 1996).

Nitric oxide is an important mediator of the inflammatory response. In the present study, we found that *in vivo* zymosan exposure followed by *ex vivo* LPS stimulation produced a lower than expected LPS induction of nitric oxide production from AM (Fig. 2). That is, LPS-induced nitric oxide production by AM was decreased by in vivo pretreatment with zymosan. A similar effect was observed with NO-dependent chemiluminescence in rats pretreated with zymosan and then exposed to LPS (Table 2). The NO-dependent chemiluminescence was conducted to measure nitric oxide synthase inhibitable chemiluminescence by using a NOS inhibitor, L-NAME (Dikshit *et al.,* 1996). The above results suggest that responsiveness of AM to LPS is decreased in rats pretreated with zymosan.

Of interest is why pretreatment of rats with zymosan did not lower the breathing rate response to subsequent LPS exposure. Breathing rate enhancement has been shown to be strongly associated with PMN pulmonary infiltration (Castranova *et al.,* 1988). Indeed, depletion of circulating PMN by cyclophosphamide treatment resulted in a 75% reduction in breathing frequency enhancement and a 99% decrease in PMN pulmonary infiltration after exposure to cotton dust compared to nondepleted rats (Castranova *et al.,* 1988). In this experiment, pretreatment with zymosan did not inhibit LPS-induced PMN pulmonary infiltration (Table 2). The reasons why zymosan pretreatment did not inhibit LPS-induced PMN pulmonary infiltration are not clear. TNF-α has been reported to be a chemoattractant for PMN (Driscoll *et al.,* 1995), and a direct relationship has been shown between TNF-α production and PMN recruitment in silica-exposed rats (Driscoll and Guthrie, 1997). In the present study, BALF TNF-α levels were lower than expected in zymosan-pretreated then LPS-exposed rats (Fig. 1). This lower than expected LPS stimulation of TNF-α production did not inhibit PMN infiltration. It may be that zymosan-induced PMN infiltration is too great to modulate (2.7×10^6 PMN in PBS-IT controls compared to 17.7×10^6 PMN after zymosan-IT). Zymosan has been reported to stimulate leukotriene B_4 (LTB_4) and C_4 (LTC_4) production in human monocytes (Czop and Austen, 1985) and blood levels of platelet activating factor (PAF) in mice (Miotla *et al.,* 1998). These are potent chemoattractants for PMNs. High levels of LTB_4 and PAF may set the stage for further PMN infiltration in response to LPS. In contrast to the present intracheal instillation study with rats, Robinson *et al.,* (1996) reported that preexposure to zymosan inhibited PMN infiltration and the breathing rate increase in response to subsequent LPS exposure. However, this was an inhalation exposure study with guinea pigs. Therefore, the conflicting results of these two studies may be due to species differences and/or differences in exposure route. Indeed, since the delivered dose of zymosan was higher after IT exposure than following inhalation, greater inflammation was noted after IT exposure to zymosan. As stated above, the high inflammatory state may have primed the rats to respond to subsequent LPS exposure.

In summary, the interaction of zymosan and LPS on the pulmonary response of rats was investigated in three different exposure groups, i.e., zymosan treatment prior to LPS exposure, zymosan treatment after LPS exposure, and simultaneous zymosan and LPS exposure. A lower than expected LPS-induced pulmonary response was observed only with zymosan pretreated rats. The increased breathing rate and PMN infiltration in response to LPS were not affected by pretreatment with zymosan. However, LPS induction of other pulmonary indicators such as LDH activity, albumin level, pulmonary TNF-α, and alveolar macrophage production of reactive oxidant species was significantly lower than expected after zymosan pretreatment. Therefore, pretreatment of rats with a particulate form of 1→3-β-glucans appears to inhibit the overall pulmonary response to LPS stimulation. These result suggests that complex interaction of components may exists in exposure to organic dusts. Therefore, hazard may not be defined by measuring endotoxin or 1→3-β-glucans alone.

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