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# ACUTE INFLAMMATION AND RECOVERY IN RATS AFTER INTRATRACHEAL INSTILLATION OF A 1→3-β-GLUCAN (ZYMOSAN A)

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*Although endotoxin is a known potent stimulant of inflammatory responses, the magnitude of pulmonary response following exposure to various organic dusts does not always correlate with endotoxin content of the dusts alone. Other components, such as 1→3-β-glucans, derived from the inner cell wall of yeasts and fungi, have been implicated in organic dust toxic syndrome. However, animal studies report conflicting results concerning the inflammatory potency of 1→3-β-glucan. In this experiment, the pulmonary reaction of rats to 1→3-β-glucan (zymosan A) exposure was assessed. Male Sprague-Dawley rats were exposed via intratracheal instillation (IT) to zymosan A (dose range 0–5 mg/kg body weight). Rats were sacrificed 1–7 d postexposure and the following pulmonary responses were monitored: (1) breathing frequency, (2) differential cell counts of bronchoalveolar lavage (BAL) cells, (3) chemiluminescence (CL) as a measure of alveolar macrophage activation, (4) nitric oxide production by alveolar macrophages, (5) albumin levels, and (6) lactate dehydrogenase (LDH) activity in the first acellular lavage fluid. Upon challenge with zymosan A, rats exhibited a dose-dependent pulmonary response at 1 d post IT that was significantly higher than the control level at a dose of 1–2.5 mg/kg body weight for each of these pulmonary parameters. Post-IT enhancement of breathing frequencies and polymorphonuclear leukocytes (PMN) obtained by BAL both correlated very well with zymosan A concentration ($r^2$ = .95 and .99, respectively). Elevation of albumin levels and LDH activity of the acellular BAL fluid also correlated ($r^2$ = .80) with the dose of zymosan. The recovery from a single intratracheal administration of zymosan A (2.5 mg/kg body weight) was monitored over 7 d. PMN and CL showed significant recovery from d 1 level by 3 d postexposure. Breathing frequencies and nitric oxide production showed significant recovery from d 1 level by 4 d postexposure. A good correlation ($r^2$ = .8) between recovery of PMN in BAL, CL, or nitric oxide production and the days postexposure was observed.*

1→3-β-Glucans and endotoxin (lipopolysaccharide, LPS) have been considered to be two of the most common airborne etiological agents in organic dust-induced respiratory disease (Zejda & Dosman, 1993; Young et al., 1998). Endotoxin has been studied extensively for its role in occupational lung diseases (Jacobs, 1989; Pratt et al., 1994; Rylander & Jacobs,

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1994). However, there is relatively less information available for 1→3-β-glucans. This may be because tools to specifically measure 1→3-β-glucans were not available until recent years. Endotoxin and 1→3-β-glucans share many similarities: (1) Both are cell wall constituents, (2) they have polysaccharides in their structure, and (3) both are positive in the *Limulus* amebocyte lysate (LAL) assay. In spite of their similarities, the impetus for their study has been from two different directions. Studies of endotoxin have focused on adverse effects, while studies of 1→3-β-glucan have focused on potential beneficial effects (Di Luzio, 1983). Only recently have researchers linked 1→3-β-glucan with respiratory disease (Rylander et al., 1992).

Endotoxin is a known potent stimulant of inflammatory responses. High endotoxin levels, measured by the LAL assay, often indicate the inflammatory potential of a dust. However, measured endotoxin content in organic dusts does not always correlate well with inflammatory indicators (Castranova et al., 1991). Thus other components of organic dusts appear to contribute to the observed symptoms. 1→3-β-Glucans often coexist with endotoxin in organic dusts; like endotoxin, 1→3-β-glucans also test positively in LAL assay (Kakinuma et al., 1981; Cooper et al., 1997; Nakao et al., 1997; Vassallo & Limper, 1999). It is possible that 1→3-β-glucans also contribute to the inflammatory responses of the lung. However, the role of 1→3-β-glucans in pulmonary inflammation is still not clear. A potentiating action of 1→3-β-glucans on the allergic response to ovalbumin has been reported recently (Ormstad et al., 2000). 1→3-β-Glucans have also been reported to be potent immune stimulating agents (Riggi & Di Luzio, 1961; Di Luzio, 1983). Occupational exposure to 1→3-β-glucans can be found in a variety of settings, such as waste treatment, agriculture, wood pulping, bakeries, etc. Exposure to 1→3-β-glucans has also been associated with indoor air contamination, particularly in buildings improperly insulated against the wet ground or when leakage through roofs or water pipes has occurred.

In reviewing the linkages between the 1→3-β-glucans and their potent effects, there is clearly a need to investigate the adverse pulmonary effects of 1→3-β-glucan further. Few papers have focused on the toxicity of 1→3-β-glucans. Animal studies report conflicting results concerning the inflammatory potency of 1→3-β-glucan. Inhalation of curdlan, a 1→3-β-glucan from soil bacteria, by guinea pigs did not result in pulmonary inflammation in either acute (Fogelmark et al., 1992) or chronic (Fogelmark et al., 1994) experiments. However, inhalation of a purified 1→3-β-glucans product, zymosan A, resulted in an acute inflammatory response (Robinson et al., 1996). A direct comparison between the results was not possible because of the use of different types of glucans. However, Schuyler et al. (1998) showed that intratracheally (IT) instilled curdlan produced increased pulmonary abnormalities. This suggests that appropriate conditions may be necessary to observe the inflammatory response.

Our laboratory has established an animal model for the study of pulmonary reactions to organic dust exposures (Castranova et al., 1996). In order to determine the inflammatory potential of 1→3-β-glucans, the acute inflammatory response to intratracheal instillation of zymosan A was examined in rats. The following indicators of inflammation and pulmonary damage were monitored: (1) elevated breathing frequencies, (2) an increase of polymorphonuclear leukocytes in differential cell counts of bronchoalveolar lavage cells, (3) elevated alveolar macrophage chemiluminescence, (4) increased nitric oxide production by alveolar macrophage-enriched cell preparations, (5) an increase in albumin concentrations in the first acellular lavage fluid, and (6) lactate dehydrogenase (LDH) activity in the first acellular lavage fluid.

## MATERIALS AND METHODS

### Preparation of Zymosan A Samples

Zymosan A, from baker's yeast, was obtained from Sigma Chemical Company (St. Louis, MO). The majority of particles were between 2–5 μm in diameter, which is in the range of respirable particles. The particle size distribution in suspension was about the same as in inhalation studies (Robinson et al., 1996). Zymosan A was freshly prepared as an aqueous suspension before every experiment.

### Animals and Treatments

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 d before use. Food and water were given ad libitum. Before intratracheal instillation, rats were lightly anesthetized with an intraperitoneal injection of 0.6 ml of 1% (w/v) sodium methohexital (Eli Lilly Co., Indianapolis, IN). The zymosan A suspension was quickly instilled into the trachea. Rats received a single dose of the indicated amount (in mg/kg body weight) of zymosan A. Control animals were instilled with the sterile saline vehicle only, in a volume of 0.26 ml/rat. For the dose-response experiments, rats were sacrificed at 1 d post-IT. For the recovery study, intratracheally instilled rats were sacrificed at 1, 2, 3, 4, or 7 d postexposure.

### 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 from 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 breathing frequencies, a rat was placed in the flow plethysmograph, equilibrated with 10% $CO_2$, and the rat's 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 Bronchoalveolar Lavage Fluid

Rats were anesthetized with 0.7 ml 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 saline solution (PBS: 145 m*M* NaCl, 5 m*M* KCl, 1.9 m*M* $NaH_2PO_4$, 9.35 m*M* $Na_2HPO_4$, and 5.5 m*M* glucose; pH 7.4) at a volume of 6 ml for the first lavage and 8 ml for subsequent lavages. A total of 80 ml bronchoalveolar lavage fluid (BALF) was collected from each rat and centrifuged at 800 × g for 10 min at 4°C. The supernatant from the first lavage was saved for analysis of albumin content and LDH activity. Supernatants from other lavages were decanted and discarded. All cell pellets from an individual rat were combined as the bronchoalveolar lavage (BAL) cells, resuspended in 1 ml HEPES-buffered solution (145 m*M* NaCl, 5 m*M* KCl, 10 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 1 m*M* $CaCl_2$, and 5.5 m*M* D-glucose; pH 7.4), and placed on ice. Aliquots of cell suspensions from each rat were taken for determination of total cell and differential cell counts using a Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL). The remaining BAL cells were used for primary cell culture 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 the damage/permeability of alveolar–capillary barrier. The activity of lactate dehydrogenase (LDH), a cytosolic enzyme, in the first acellular BAL fluid was assayed to evaluate general cell 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 bromcresol green (albumin BCG diagnostic kit, Sigma Chemical Company, St. Louis, MO) and expressed as milligrams per milliliter BAL fluid. LDH activity was measured by the formation of NADH and expressed as units per liter BALF using Roche Diagnostic reagents and procedures (Roche Diagnostic Systems).

### Alveolar Macrophage Chemiluminescence

The alveolar macrophage (AM) chemiluminescence (CL) assay was conducted in a 0.25 ml reaction mixture of HEPES-buffered solution. Rest-

ing AM CL was determined by incubating $0.25 \times 10^6$ AM cells at 37°C for 20 min, then adding 0.008 mg% (w/v) luminol (Sigma Chemical Company, St. Louis, MO), 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 unopsonized zymosan (Sigma Chemical Company, St. Louis, MO), which was added immediately prior to measure 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 m*M* *N*-nitro-L-arginine methyl ester HCl (L-NAME, Sigma Chemical Company, St. Louis, MO) to the cells prior to preincubation. NO-dependent CL was calculated as the difference between zymosan-stimulated CL measurements with and without L-NAME.

### Primary BAL Cell Culture

The BAL cells from each rat were suspended in RPMI-1640 culture medium (RPMI, Sigma Chemical Co.) containing 2 m*M* glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10% heat-inactivated fetal bovine serum. Aliquots of cell suspensions from each rat, adjusted to $0.5 \times 10^6$ 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 (37°C and 5% $CO_2$). The nonadherent BAL cells were then removed by rinsing the monolayers three times with RPMI media. These AM-enriched preparation were incubated (37°C and 5% $CO_2$) in fresh RPMI medium for 24 h. The AM-conditioned media were collected and the supernates were analyzed for nitric oxide or stored at –70°C until the time for analysis of nitric oxide.

### Determination of Nitric Oxide

Nitric oxide was determined as nitrite ($NO_2^-$) with Griess reagent as described by Ljungman et al. (1998). Nitrite concentrations were determined using a standard curve prepared from sodium nitrite with a linear range from 1.6 to 206 μ*M*. Three replicates were done for each sample in the experiment.

### Statistical Analysis

All data were expressed as the mean ± standard error of mean (SEM). Statistic analysis was performed using SigmaStat v2.0. software (Jandel Corporation). The significance was set at $p \leq .05$. A *t*-test was used to compare treatment and control groups. For multiple comparison, a one-way analysis of variance (ANOVA) for comparing several treatment groups with one control was used. Linear regression or Pearson correlation analysis was used to calculate the correlation coefficient.

## RESULTS

### Endotoxin

To exclude the possibility that the results were due to contamination with LPS, the aliquots of zymosan suspension used for the instillation were centrifuged and the supernates analyzed for endotoxin content using an LAL (from Whittaker Bioproducts) reagent. The endotoxin content of zymosan suspensions was low, ranging from 9.2 to 28.6 EU/ml. Therefore, the amount of endotoxin that rats received ranged from 2.39 to 7.43 EU. This dose is equivalent to 0.239 to 0.743 ng endotoxin. A similar level of endotoxin was found in control rats instilled with PBS, which did not affect breathing rate (Figure 1) or cause significant PMN infiltration ($2.85 \pm 0.24 \times 10^6$ cells) from the non-PBS instilled level (Figure 2).

### Dose Response to Zymosan Exposure

**Breathing Frequencies** The breathing frequency measurement is a noninvasive method for assessing pulmonary response in rats. Instillation of dust

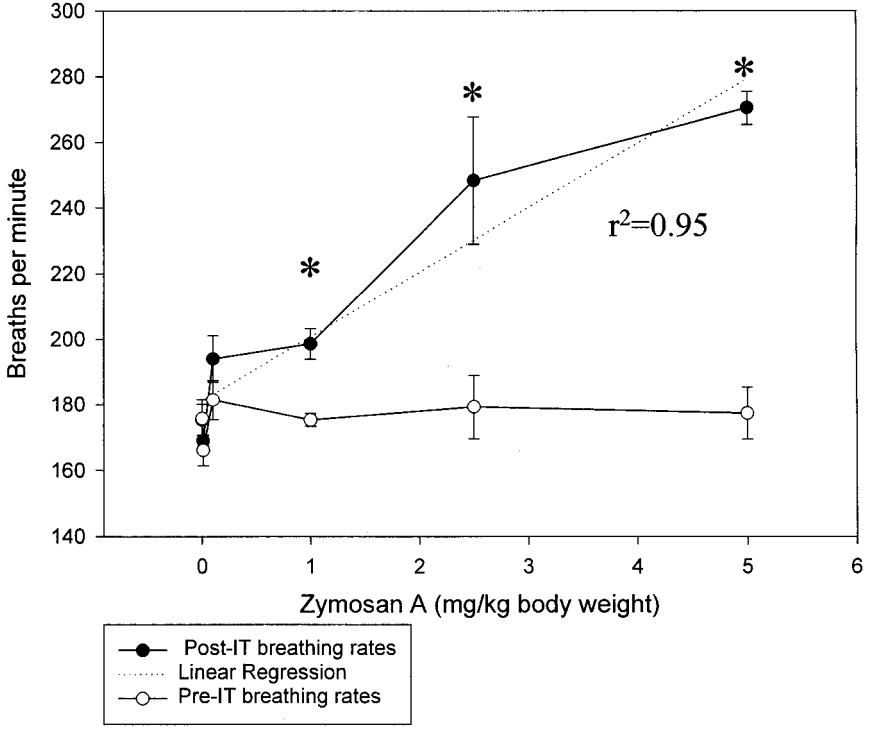


**FIGURE 1.** Breathing frequencies of rats in 10% $CO_2$ 1 d after IT instillation with various doses of zymosan. Asterisk indicates a significant increase above the pre-IT level. The correlation coefficient ($r^2$) equals .95 between zymosan and 1 d post-IT breathing frequencies. Values are means ± SEM of three to six rats.

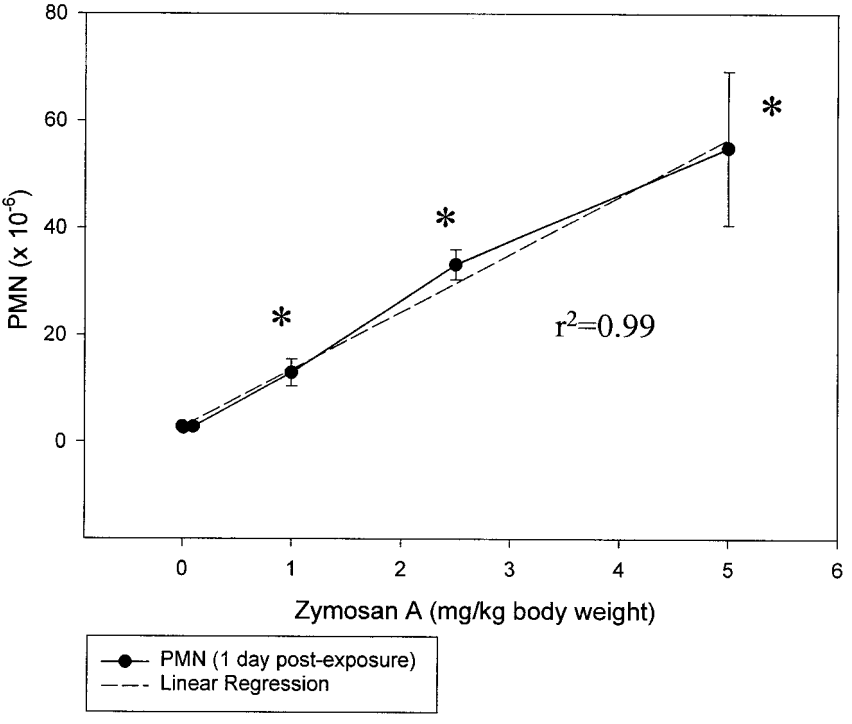


**FIGURE 2.** Polymorphonuclear leukocytes (PMN) obtained by BAL of rats 1 d after IT instillation with various doses of zymosan A. Asterisk indicates a significant increase above the control PMN level. Values are means ± SEM of three to six rats. $r^2$ = .99 between zymosan A and 1 d post-IT PMN cells.

particles has been shown to cause a change in respiratory rate (Robinson et al., 1997; Porter et al., 1999). Breathing frequencies were determined immediately prior to the IT administration of zymosan A and immediately prior to sacrifice at 1 d post-IT (Figure. 1). A zymosan concentration of 1 mg/kg resulted in a statistically significant increase in breathing rate at 1 d post-IT rate (30 breaths/min) compared to the controls (pre-IT rates). The breathing frequencies increased further after exposure to 2.5 and 5 mg/kg zymosan. The increase in post-IT breathing frequencies exhibited a good correlation with the IT dose of zymosan (correlation coefficient $r^2$ = .95).

**Cell Differentials** Bronchoalveolar lavage (BAL) was used to assess the effect of exposure to zymosan particles on pulmonary inflammatory responses by monitoring neutrophil infiltration as determined by the number of polymorphonuclear leukocytes (PMN) harvested by BAL. In control rats, the majority of BAL cells are AM cells. However, IT instillation of zymosan A induced PMN infiltration into the lung airspace, resulting in a higher yield of PMN in BAL. PMN recruitment is a very sensitive indicator of an inflammatory response. There was a dose-dependent increase in lavagable PMN 1 d

after zymosan A exposure (Figure 2). At 1 mg/kg zymosan A, the number of PMN ($13.2 \times 10^6$ cells/rat) obtained by BAL exceeded the number of lavagable AM (data not shown). Figure 2 shows that PMN infiltration correlated well with zymosan A concentration delivered IT ($r^2 = .99$). Alveolar macrophage (AM) cells counts in the BAL did not differ significantly with zymosan A exposure (data not shown).

**Albumin Concentration** Albumin concentration was measured in first acellular lavage fluid as an indicator of damage to the alveolar blood–gas barrier. Albumin levels in BAL fluid increased in a dose-dependent fashion 1 d after IT treatment with zymosan (Table 1). This increase was statistically significant at 2.5 mg/kg zymosan, where the BAL albumin level was almost twice that of control. Albumin levels of BAL fluid correlated well with zymosan A exposure levels ($r^2 = .801$).

**LDH Activity** Pulmonary cell damage resulting from zymosan A exposure was evaluated by measuring lactate dehydrogenase (LDH) activity in the acellular first BAL fluid. Table 1 shows a dose-dependent increase in pulmonary cell damage 1 d after zymosan A exposure. A statistically significant difference was observed at 2.5 mg/kg zymosan, where LDH activity increased by 68% above control BAL. LDH activity correlated well with zymosan A exposure concentration ($r^2 = .80$).

**Alveolar Macrophage Chemiluminescence** Chemiluminescence (CL) generated by alveolar macrophages (AM) was monitored to assess the activation of these pulmonary phagocytes due to IT exposure to zymosan particles. Table 1 shows the dose-dependent increase in CL 1 d after IT instillation of zymosan. Statistically significant differences were observed after IT exposure to 1 mg/kg and 2.5 zymosan A, 4.1- and 4.9-fold increases, respectively (Table 1). At the highest zymosan exposure (5 mg/kg), CL was not significantly different from the control level. This could be due to the high cytotoxicity observed at this IT dose of zymosan A, resulting in a decreased ability of macrophages to generate reactive species in response to particulate exposure.

**TABLE 1.** Pulmonary Response to Zymosan Exposure

| Zymosan (mg/kg) | Albumin (mg/ml) | LDH activity (U/L) | Chemiluminescence [a] (cpm $\times 10^{-5}$/0.25 $\times 10^6$ AM/15 min) |
|---|---|---|---|
| Control | 0.22 ± 0.03 | 56.6 ± 4.4 | 2.6 ± 1.1 |
| 0.01 | 0.20 ± 0.02 | 47.3 ± 4.2 | 6.7 ± 4.9 |
| 0.1 | 0.24 ± 0.06 | 47.0 ± 4.2 | 3.6 ± 1.4 |
| 1 | 0.32 ± 0.06 | 82.0 ± 13.8 | 10.7 ± 2.6[b] |
| 2.5 | 0.41 ± 0.03[b] | 95.0 ± 6.5[b] | 12.7 ± 1.2[b] |
| 5 | 0.66 ± 0.17[b] | 108 ± 13.3[b] | 8.2 ± 4.0 |

*Note.* Values are expressed as means ± SEM.
[a]Zymosan-stimulated chemiluminescence.
[b]Significantly higher than control level.

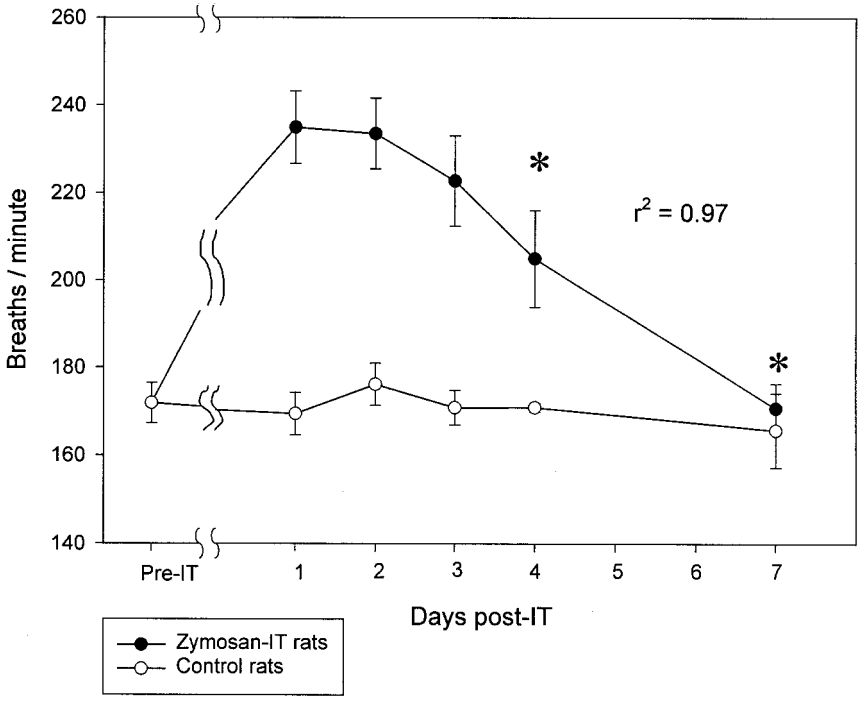


**FIGURE 3.** Recovery of breathing frequency after zymosan A exposure. Breathing frequencies of rats were measured at 1–7 d after a single IT exposure to zymosan A (2.5 mg/kg). Asterisk indicates a significant decrease from the 1 d post-IT level; $r^2$ = .972 between breathing frequencies and days after zymosan A exposure. Values are means ± SEM of 8 to 28 rats.

### Recovery From Zymosan A Exposure

**Breathing Frequencies** Figure 3 shows that although the breathing frequency of exposed rats increased 1 d after zymosan instillation (2.5 mg/kg), breathing frequency tended to return toward normal on subsequent days postexposure. At d 4 postexposure, breathing frequency had recovered significantly from the d 1 level. This recovery was even greater at 7 d postexposure. The decrease in post-IT breathing frequencies correlated well with days post-IT ($r^2$ = .97).

**Cell Differentials** In Figure 4, the recovery from inflammation was suggested as the decrease in PMN harvested by BAL with increasing time after a single IT exposure to zymosan A (2.5 mg/kg). Day 1 post-IT had the highest PMN level ($30 \times 10^6$ cells/rat). At d 3 postexposure, the PMN level was significantly decreased from the d 1 level and approached the control level by 7 d after zymosan A instillation. Recovery from inflammation (PMN infiltration) correlated well with days post-IT ($r^2$ = .869).

**Albumin Concentration** Table 2 shows the time course of recovery from lung injury caused by a single instillation of zymosan A (2.5 mg/kg)

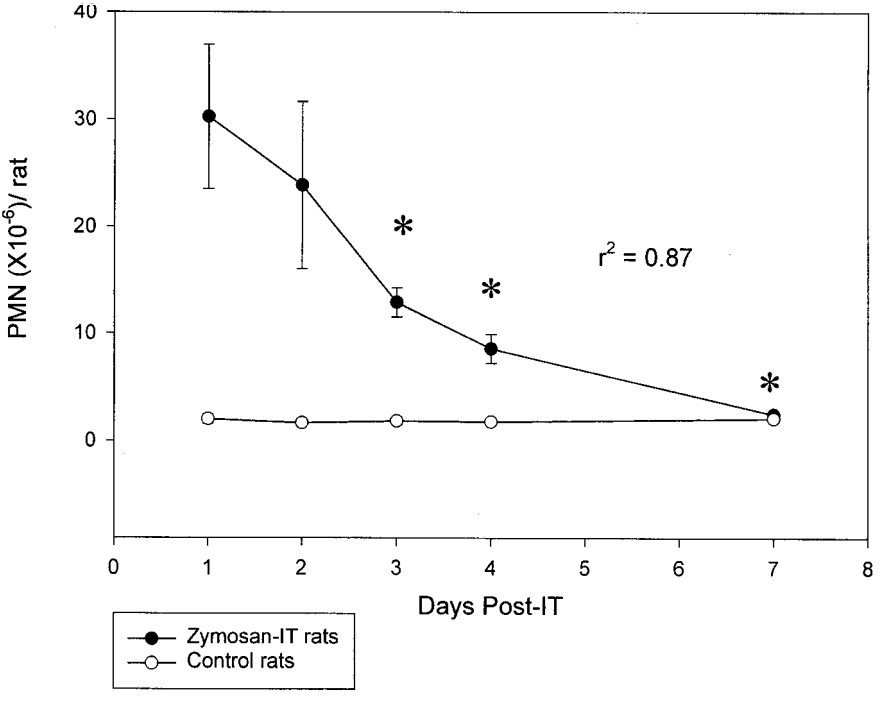


**FIGURE 4.** Recovery of PMN levels after zymosan A exposure. PMN harvested by BAL of rats 1–7 d after a single IT exposure to zymosan A (2.5 mg/kg). Asterisk indicates a significant decrease from the 1 d post-IT level; $r^2$ = .869 between PMN and days after zymosan A exposure. Values are means ± SEM of 8 to 10 rats.

in rats. Unlike PMN infiltration or breathing frequency, the albumin response remained significantly higher than control from d 1 to d 4 post-IT, reaching a maximum albumin level at d 3 (4.4 times control). Only at d 7 postinstillation did the albumin level significantly decrease from the d-1 level, indicating significant recovery from injury.

**TABLE 2.** Recovery of Pulmonary Response After Zymosan Exposure

| Time (d) | Albumin (mg/ml) | LDH activity (U/L) | Chemiluminescence [a] | NO-dependent chemiluminescence | Nitric oxide (μ*M*) |
|---|---|---|---|---|---|
| Control | 0.13 ± 0.01 | 26.75 ± 2.2 | 2.37 ± 0.56 | 0.82 ± 0.22 | 4.5 ± 0.9 |
| 1 | 0.42 ± 0.05 | 112.22 ± 15.8 | 17.72 ± 5.36 | 5.01 ± 3.72 | 166.4 ± 18.4 |
| 2 | 0.38 ± 0.09 | 187.17 ± 24.1 | 17.25 ± 2.68 | 4.56 ± 1.67 | 164.6 ± 17.1 |
| 3 | 0.57 ± 0.03 | 177.71 ± 39.8 | 8.08 ± 1.69[b] | 2.76 ± 0.91 | 142.6 ± 30.0 |
| 4 | 0.41 ± 0.05 | 109.63 ± 15.6 | 2.90 ± 0.3[b] | 0.85 ± 0.26[b] | 102.9 ± 13.1[b] |
| 7 | 0.15 ± 0.01[b] | 31.75 ± 5.5[b] | 0.55 ± 0.07[b] | 0.08 ± 0.05[b] | 5.0 ± 3.2[b] |

*Note.* Values are expressed as means ± SEM.
[a]Zymosan-stimulated chemiluminescence (cpm × $10^{-5}$/0.25 × $10^6$ AM/15 min).
[b]Significantly decreased from d-1 level.

**LDH Activity** Similar to the recovery time course for albumin, LDH activity of the acellular BAL fluid was the highest at d 2 post-IT (7 times greater than control) and remain significantly above control from 1 to 4 d post zymosan instillation (2.5 mg/kg). Only at d 7 was the LDH activity significantly decreased from the d-1 level, indicating a significant recovery from cell damage.

**Alveolar Macrophage Chemiluminescence and NO-Dependent Chemiluminescence** Table 2 shows the time course of CL and NO-dependent CL response of alveolar macrophages after a single IT instillation of 2.5 mg/kg zymosan A. The highest AM CL (7.5-fold above control) and NO-dependent CL (6-fold increase than control) was observed at 1 and 2 d post-IT. At d 3 post-IT, the CL level was significantly decreased from d-1 level, indicating a significant recovery from inflammation. NO-dependent CL was significant decreased from d-1 level at d 4. The decrease of AM CL correlated well with days post IT ($r^2$ = .817).

**Nitric Oxide Production by AM** Nitric oxide production was measured to assess the potential inflammatory activation of alveolar macrophages (AM) due to exposure to zymosan A particles (2.5 mg/kg). Table 2 shows the time course of the induction of nitric oxide release from AM, measured by the Griess reaction. Nitric oxide release was the highest at d 1 post-IT. At 4 d post-IT, nitric oxide production was significantly decreased from the d-1 level, indicating significant recovery from inflammation. This recovery was nearly complete at 7 d post-IT. The decrease of nitric oxide production correlated well with days post IT ($r^2$ = .816).

## DISCUSSION

1→3-β-Glucans are a major component of the fungal cell wall. In the present study, a purified 1→3-β-glucan, zymosan A, was used to simulate the effect of workplace exposure to fungal products. Zymosan A-induced acute pulmonary inflammatory responses in rats were characterized by the following changes: (1) an increase in breathing frequency, (2) PMN infiltration into the airspaces, (3) an increase of the albumin concentration and LDH activity in the first BAL fluid, (4) enhancement of particle-stimulated alveolar macrophage chemiluminescence, and (5) an increase in nitric oxide production by alveolar macrophages. The induction was dose dependent, while recovery from inflammatory responses was time dependent.

A significant inflammatory/damage process was observed in zymosan A-instilled rats. Increased LDH activity indicates that lung cells were damaged as a result of zymosan's cytotoxic properties or by inflammatory products (reactive species produced by AM). Albumin is a marker of a deterioration of the blood–gas barrier in the lung. Data indicate that the production of ROS and NO from AM is enhanced after zymosan A exposure. Elevated levels of these oxidants may result in direct damage to the alveolar–capillary barrier. This increase in vascular permeability also may

result from the migration of neutrophils into lung. One of the major indicators in inflammation is the infiltration of PMN. Milanowski et al. (1995) found that microbial products were able to attract both AM and PMN directly in a dose-dependent manner, and that the exposure of cultured AM to microbial agents increased the production of chemoattractants for AM and PMN. Czop and Austen (1985) found that leukotrienes were produced by human monocytes upon stimulation of their β-glucan receptor during phagocytosis. Leukotriene $B_4$ is a potent chemoattractant for PMNs. PMN may play a significant role in the increase of breathing frequencies in response to microbial products. It has been shown that rats depleted of PMN by cyclophosphamide treatment exhibited a 75% reduction in breathing frequency enhancement and a 99% decrease in PMN infiltration after exposure to cotton dust compared to nondepleted rats (Castranova et al., 1988). In our study, a 15-fold increase of PMN was associated with the maximal increase in breathing rate at d 1 post-IT. There was a correlation ($r^2$ = .89) between the recovery of PMN and the return of breathing rate to normal after zymosan A exposure.

In the present investigation, activation of AM cells was assessed by monitoring chemiluminescence and nitric oxide production. The results show a significant enhancement of reactive oxygen and reactive nitrogen production from macrophages harvested from zymosan-exposed rats compared to controls. L-NAME, at a final concentration of 1 m*M*, was added to determine the fraction of CL which was due to reactive nitrogen species. The data indicate that about one-third of the CL was eliminated by treatment of cells with this inhibitor of nitric oxide synthase. Our results are supported by other reports that 1→3-β-glucans induced macrophage cells to secrete reactive oxygen (Kilgore et al., 1997) and reactive nitrogen intermediates (Ljungman et al., 1998).

Intratracheal exposure to 1→3-β-glucan induced acute pulmonary inflammation in the present study. Although the IT route of exposure was chosen to establish a dose response and the time course for the inflammatory response of rats to zymosan A, inhalation of zymosan A has been shown to cause a similar pulmonary response (Robinson et al., 1996). The magnitude of PMN infiltration was comparable in both experiments. In the inhalation study, guinea pigs were exposed to various concentrations of zymosan aerosol for 4 h, and PMN were harvested from BAL after 18 h postexposure. The PMN infiltration in response to an IT zymosan dose of 1 mg/kg was comparable to that after inhalation of 6 mg/m$^3$ for 4 h (PMN ~15 × $10^6$ cells). At this concentrations, the estimated inhaled dose of zymosan was 0.15 mg/guinea pig, which is comparable to 0.26 mg/rat in the present IT experiment. An IT zymosan dose of 2.5 mg/kg resulted in comparable inflammation as 23 mg/m$^3$ for 4 h by inhalation (PMN ~35 × $10^6$ cells); that is, the estimated inhalation dose was 0.58 mg/guinea pig, which is comparable to 0.65 mg/rat in the IT experiment. By using IT instillation, we were able to deliver a relatively large dose in a short period of

time, which is one of the advantages of IT instillation over inhalation (Kennedy et al., 2000). Inhalation of another 1→3-β-glucan, curdlan, did not result in pulmonary inflammation with guinea pigs in either acute (Fogelmark et al., 1992) or chronic (Fogelmark et al., 1994) experiments. This maybe due to differences in the biological activity of different 1→3-β-glucans. The biological activity of 1→3-β-glucans is thought to be related to three factors: molecular conformation, the degree of branching (DB), and the molecular weight (MW) (Bohn & BeMiller, 1995). Studies have shown that high-MW (100,000–200,000 g/mol) glucans with a DB of 0.20–0.33 are most active (Bohn & BeMiller, 1995). Although curdlan has a high MW, it doesn't exhibit (1→6) branching in its structure (DB = 0) (Ohno et al., 1990). In comparison, zymosan A has a high MW and has a DB of approximately 0.03–0.2 (Manners et al., 1973). Therefore, zymosan is generally considered to be more potent than curdlan.

Exposure to 1→3-β-glucans has been associated with airway inflammation in several occupational settings, including house waste collection (Thorn et al., 1998), composting (Douwes et al., 2000), sewage treatment (Rylander, 1999), and paper processing (Rylander et al., 1999). The following airborne 1→3-β-glucans levels have been reported: compostable waste glucan levels ranging from 10.8 to 36.4 ng/m$^3$ (Thorn et al., 1998), composting from 0.36 to 4.85 μg/m$^3$ (Douwes et al., 2000), and paper mills from 4 to 366 ng/m$^3$ (Rylander et al., 1999). Although these workplace 1→3-β-glucans exposure levels are much lower than those for the current IT experiment, exposure duration is often longer and associated with airway inflammation. Therefore, these data support the hypotheses that 1→3-β-glucans have a direct effect on inducing pulmonary inflammation in rats. The inflammatory potential of glucans may be a significant factor in explaining why the magnitude of pulmonary response following exposure to various organic dusts does not always correlate with endotoxin content of the dusts alone.

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