Brief Communication

Zymosan priming protects rats against pulmonary oxygen toxicity: characterization of the model

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Abstract

This laboratory previously reported that zymosan priming protects rats against pulmonary oxygen toxicity. That study used a standard priming protocol (3 daily intravenous injections [15 mg zymosan/rat] with 2 days' rest) before hyperoxia. This study confirms that report and more fully characterizes the zymosan priming model. Three studies were conducted to establish the (1) effects of dosage, (2) role of duration of rest period between injections and hyperoxia and (3) importance of injection number, on protection by zymosan priming. Rats were exposed to >95% oxygen for 52 h or room air and acute lung injury was quantitated using standard methods. Lung injury decreased ($P < 0.05$ versus saline controls) in all groups of zymosan-primed rats (3 daily intravenous injections [1–15 mg zymosan/rat] with 2 days' rest before hyperoxia). Although the differences between zymosan-primed groups were not statistically significant, protection (as indicated by decreasing mean values of measured parameters of lung injury) increased with dosage. A one-day rest after injections was sufficient to partially protect zymosan-primed rats from hyperoxia (some measured parameters in the zymosan-primed group differed significantly from comparable values in the Saline group), but full protection (all measured parameters within a group differed significantly from Saline values) was not produced until rats received two days' rest before hyperoxia. Finally, one or two zymosan treatments produced partial protection against oxygen toxicity but three injections were needed to produce full protection. In conclusion, this study found that the standard priming protocol (3 zymosan injections with 2 days' rest before hyperoxia) was the most effective in protecting rats against hyperoxia.

Keywords: acute lung injury, adjuvant, animal models, hyperoxia, oxygen toxicity, Toll-like receptors, zymosan, zymosan priming

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Introduction

Zymosan is a non-bacterial, endotoxin-free substance derived from the yeast cell wall. When administered as a priming agent (three daily intravenous injections with two days' rest before hyperoxia), zymosan induces adjuvancy[1–3] where macrophages become hypersensitive and release tumor necrosis factor and interleukin 1 at reduced dosages of stimulation.[4–7]

Previously, we observed adjuvancy in a mouse model of oxygen toxicity when zymosan priming caused a 10-fold reduction in the amount of endotoxin required to extend survival in hyperoxia.[8] In addition, the dosage of endotoxin providing optimal protection against oxygen toxicity in unprimed mice (20 μg endotoxin/mouse) became a lethal dose when given to zymosan-primed animals (primed mice died from endotoxemia and not oxygen toxicity).

This laboratory previously reported that zymosan priming protects rats from pulmonary oxygen toxicity.[9] The extent of protection against hyperoxia is similar to that afforded by endotoxin, but there are major differences between protection by endotoxin and zymosan. For example, endotoxin protects rats against oxygen toxicity when given by either intraperitoneal or intravenous injection whereas zymosan protects only when given intravenously.[9] Also, endotoxin is protective when given as late as 36 h into oxygen exposure[10] while zymosan requires multiple injections with a two-day rest period before hyperoxia as shown in this study. These observations demonstrate that endotoxin acts quickly (more directly) while zymosan acts more slowly and may involve the immune response at some level.

The goal of the present study was to characterize the zymosan priming model and determine the importance of

dosage, duration of rest period and number of injections on protection against oxygen toxicity.

Methods

Animals

Breeder Sprague–Dawley rats were obtained from the Food and Drug Administration (Alabang, Philippines) and bred at Lung Injury Research Institute (San Carlos City, Pangasinan, Philippines). Rats were maintained, *ad libitum*, on Meg-3000 high-protein chicken feed and pigeon pellets and had access to water at all times. All procedures were approved by the Bureau of Animal Industry, Animal Welfare Division (Quezon City, Philippines) (BAI. No. AR 2008-001) and animals were in excellent health and free of respiratory infection and disease at all times.

Injection and exposure protocols

Zymosan A (No. Z4250) was obtained from Sigma Chemical Company (St Louis, MO, USA). The standard priming protocol was based on the method of Moore *et al.*[3] Accordingly, rats received three daily tail vein injections with Zymosan A in saline (3 mL volume) followed by two days' rest before exposure to hyperoxia (>95% oxygen at sea level pressure). A total of 41 rats were used for the three study groups and there were 12 different treatment groups.

Study 1 (dose–response, 29 rats): Twenty-three rats received standard priming protocol treatment with 0, 1, 5, 10, 12 or 15 mg zymosan/rat (saline, Z-1, Z-5, Z-10, Z-12 and Z-15 groups, Table 1) and exposed to 52 h hyperoxia. Two of five rats receiving 15 mg zymosan died after treatment. Because of toxicity, the maximal dosage of zymosan was reduced to 12 mg/rat in future studies. Six other rats were exposed to room air (3 received the standard priming protocol with 12 mg zymosan/rat [Z-12/Air group] and 3 rats received no treatment, No Tr/Air, Table 1).

Study 2 (duration of rest): Six additional rats were used in Study 2 (3I,1R and 3I,5R groups with I = injections and R = rest, Table 2). Data from four of the Study 1 treatment groups (Saline, Z-12/Hyperoxia [labeled '3I,2R' in Table 2], Z-12/Air [labeled '3I,2R/Air' in Table 2] and No Tr groups) were used for data analysis in Study 2. All rats, except Saline and No Tr groups, received three priming injections (12 mg zymosan/injection) with one, two or five days' rest before hyperoxia.

Study 3 (number of injections): Six additional rats were used in Study 3 (1I,2R and 2I,2R groups, Table 3). Data from four of the Study 1 treatment groups (Saline, Z-12/Hyperoxia [labeled '3I,2R' in Table 3], Z-12/Air, [labeled '3I,2R/Air' in Table 3] and No Tr groups) were used for data analysis as in Study 2. Rats received 1–3 injections (12 mg zymosan/tail vein injection) and two days' rest before hyperoxia.

Chamber design and exposure of rats to hyperoxia

The chamber was constructed of clear Plexiglas (16 inches × 20 inches with 8-inch-high walls). The floor was layered with wood chips and color indicator CO_2 absorbent (Sodasorb, W.R. Grace and Company, Atlanta, GA, USA) and was separated from rats by a 1-inch-high wire mesh. Small holes were drilled in opposite ends of the chamber and a 6-foot-long effluent tube was inserted into the hole opposite gas entry to reduce possible backflow of room air into the chamber during hyperoxia. Immediately before exposure to hyperoxia, body weight was determined for

Table 1 Dose–response

	Hyperoxia						Air	
Group*	Saline	Z-1	Z-5	Z-10	Z-12	Z-15	Z-12	No Tr
Effusion volume (mL)	7.75[a] ± 1.31 (4)	0.79 ± 0.65 (3)	0.33 ± 0.26 (4)	0.13 ± 0.10 (4)	0.08 ± 0.06 (3)	0.01 ± 0.001 (3)	0.16 ± 0.07 (3)	0.04 ± 0.01 (3)
Effusion [protein] (g/dL)	5.90[a] ± 0.17 (4)	4.80[b] ± 0.42 (3)	4.37 ± 0.41 (4)	4.63 ± 0.52 (3)	4.17 ± 0.32 (3)	4.10 ± 0.15 (3)	3.47 ± 0.13 (3)	3.10 ± 0.25 (3)
EP/PP†	0.884[a] ± 0.02 (3)	0.646[c] ± 0.07 (3)	0.563 ± 0.04 (4)	0.581 ± 0.04 (3)	0.509 ± 0.04 (3)	0.494 ± 0.01 (3)	0.431 ± 0.03 (3)	0.416 ± 0.02 (3)
W/D‡	5.75[a] ± 0.08 (4)	5.22[c] ± 0.13 (3)	5.27[c] ± 0.04 (4)	5.21[c] ± 0.04 (4)	5.04[c] ± 0.05 (3)	4.98[c] ± 0.12 (3)	4.71 ± 0.06 (3)	4.47 ± 0.08 (3)
Hct§ (%)	64.7[a] ± 3.76 (3)	37.8 ± 1.48 (3)	34.6[b] ± 0.69 (4)	35.3[b] ± 2.06 (4)	35.5[b] ± 0.76 (3)	34.7[b] ± 0.67 (3)	30.8[b] ± 1.96 (3)	43.2 ± 1.17 (3)
LA**	5.25[a] ± 1.31 (4)	2.17 ± 0.67 (3)	1.18 ± 0.12 (4)	2.0 ± 0.46 (4)	1.33 ± 0.33 (3)	1.67 ± 0.33 (3)	2.67 ± 0.73 (3)	1.0 ± 0.001 (3)

Analysis of pleural effusion fluid, gravimetric lung weights, blood and LA in rats treated with saline or zymosan priming and exposed to normobaric hyperoxia (>95% oxygen at sea level pressure) for 52 h or room air

No Tr, no treatment; EP/PP, effusion protein/plasma protein; W/D, wet/dry; Hct, hematocrit; LA, lung appearance

*Group. All groups (except the No Tr group) received three daily intravenous injections and two days' rest before hyperoxia or air. The Saline group received 3 mL saline/injection. Z-1, Z-5, Z-10, Z-12 or Z-15 groups received 1–15 mg zymosan in 3 mL saline/injection. All values, mean ± SEM, (*n*) = animals

†Ratio of pleural effusion protein/plasma protein concentrations

‡Ratio of wet weight/dry weight of the left lung

§Postexposure hematocrit in heparinized, abdominal aorta blood

**Postexposure LA with 1 = normal and 9 = liver-like

[a]$P < 0.05$ versus the other groups by analysis of variance (ANOVA) and Student–Newman–Keuls test (SNK)

[b]$P < 0.05$ versus the No Tr group by ANOVA and SNK

[c]$P < 0.05$ versus both Air groups by ANOVA and SNK

Table 2 Duration of rest period

	Hyperoxia				Air	
Group*	Saline	3I,1R	3I,2R	3I,5R	3I,2R	No Tr
Effusion volume (mL)	7.75[a] ± 1.31 (4)	0.98 ± 0.57 (3)	0.08 ± 0.06 (3)	0.04 ± 0.02 (3)	0.16 ± 0.07 (3)	0.04 ± 0.01 (3)
Effusion [protein] (g/dL)	5.90[c] ± 0.17 (4)	5.53[c] ± 0.13 (3)	4.17[d] ± 0.32 (3)	4.23[d] ± 0.09 (3)	3.47 ± 0.13 (3)	3.10 ± 0.25 (3)
EP/PP†	0.884[a] ± 0.02 (3)	0.687[a] ± 0.03 (3)	0.509 ± 0.04 (3)	0.518 ± 0.03 (3)	0.431 ± 0.03 (3)	0.416 ± 0.02 (3)
W/D‡	5.75[a] ± 0.08 (4)	5.26[d] ± 0.10 (3)	5.04[d] ± 0.05 (3)	4.97[d] ± 0.09 (3)	4.71 ± 0.06 (3)	4.47 ± 0.08 (3)
Hct§ (%)	64.7[a] ± 3.76 (3)	38.8 ± 2.77 (3)	35.5 ± 0.76 (3)	33.5[b] ± 0.76 (3)	30.8[b] ± 1.96 (3)	43.2 ± 1.17 (3)
LA**	5.25[a] ± 1.31 (4)	2.17 ± 0.44 (3)	1.33 ± 0.33 (3)	1.2 ± 0.001 (3)	2.67 ± 0.73 (3)	1.0 ± 0.001 (3)

Analysis of pleural effusion fluid, gravimetric lung weights, blood and LA in rats treated with saline or zymosan priming and exposed to normobaric hyperoxia (>95% oxygen at sea level pressure) for 52 h or room air

No Tr, no treatment; EP/PP, effusion protein/plasma protein; W/D, wet/dry; Hct, hematocrit; LA, lung appearance

*Group. All groups (except the No Tr group) received three daily intravenous injections (I) and one, two or five days' rest (R) before hyperoxia or air. The Saline group received 3 mL saline/injection. Other groups (except the No Tr group) received 12 mg zymosan in 3 mL saline/injection. All values, mean ± SEM, (*n*) = number of animals

†Ratio of pleural effusion protein/plasma protein concentrations

‡Ratio of wet weight/dry weight of the left lung

§Hct: postexposure hematocrit in heparinized, abdominal aorta blood

**Postexposure LA with 1 = normal and 9 = liver-like

[a]$P < 0.05$ versus other groups by analysis of variance (ANOVA) and Student–Newman–Keuls test (SNK)

[b]$P < 0.05$ versus No Tr group by ANOVA and SNK

[c]$P < 0.05$ versus 3I,2R and 3I,5R groups and both Air groups by ANOVA and SNK

[d]$P < 0.05$ versus both Air groups by ANOVA and SNK

each of three rats and colonic temperatures were recorded using a rapidly responding thermometer (Model T-6000, Miller and Weber, Inc., Queens, NY, USA). The rats were then placed in the chamber and flushed with O_2 at a flow rate of 15 L/min until O_2 levels reached 95% (Time 0). The rate of gas flow was then decreased to 4 L/min, which was sufficient to keep O_2 levels >95% (MiniOx1 Oxygen Analyzer, MSA Medical Products, Pittsburgh, PA, USA) and CO_2 levels <0.015% (Beckman Medical Gas Analyzer, Model LB-2, Fullerton, CA, USA).

Table 3 Number of injections

	Hyperoxia				Air	
Group*	Saline	1I,2R	2I,2R	3I,2R	3I,2R	No Tr
Effusion volume (mL)	7.75[a] ± 1.31 (4)	3.63[a] ± 0.23 (3)	0.84 ± 0.16 (3)	0.08 ± 0.06 (3)	0.16 ± 0.07 (3)	0.04 ± 0.01 (3)
Effusion [protein] (g/dL)	5.90[b] ± 0.17 (4)	5.97[b] ± 0.03 (3)	5.13[b] ± 0.47 (3)	4.17[c] ± 0.32 (3)	3.47 ± 0.13 (3)	3.10 ± 0.25 (3)
EP/PP†	0.884[e] ± 0.02 (3)	0.827[e] ± 0.05 (3)	0.665[b] ± 0.05 (3)	0.509[c] ± 0.04 (3)	0.431[c] ± 0.03 (3)	0.416[c] ± 0.02 (3)
W/D‡	5.75[e] ± 0.08 (4)	5.84[e] ± 0.02 (3)	5.44[a] ± 0.14 (3)	5.04[a] ± 0.05 (3)	4.71 ± 0.06 (3)	4.47[a] ± 0.08 (3)
Hct§ (%)	64.7[a] ± 3.76 (3)	45.3 ± 0.88 (3)	39.5 ± 1.32 (3)	35.5 ± 0.76 (3)	30.8 ± 1.96 (3)	43.2[d] ± 0.17 (3)
LA**	5.25[b] ± 1.31 (4)	3.50 ± 0.58 (3)	4.73 ± 1.79 (3)	1.33 ± 0.33 (3)	2.67 ± 0.73 (3)	1.0 ± 0.001 (3)

Analysis of pleural effusion fluid, gravimetric lung weights, blood and LA in rats treated with saline or zymosan priming and exposed to hyperoxia (>95% oxygen at sea level pressure) for 52 h or room air

No Tr, no treatment; EP/PP, effusion protein/plasma protein; W/D, wet/dry; Hct, hematocrit; LA, lung appearance

*Group. The Saline group received 3 mL saline/injection (I). Other groups (except the No Tr group) received 12 mg zymosan in 3 mL saline/injection and two days' rest (R) before hyperoxia or air. All values, mean ± SEM, (*n*) = animals

†Ratio of pleural effusion protein/plasma protein concentrations

‡Ratio of wet weight/dry weight of the left lung

§Hct: postexposure hematocrit in heparinized, abdominal aorta blood

**Postexposure LA with 1 = normal and 9 = liver-like

[a]$P < 0.05$ versus other groups by analysis of variance (ANOVA) and Student–Newman–Keuls test (SNK)

[b]$P < 0.05$ versus both 3I,2R groups and No Tr group by ANOVA and SNK

[c]$P < 0.05$ versus Saline, 1I,2R and 2I,2R groups by ANOVA and SNK

[d]$P < 0.05$ versus both 3I,2R groups by ANOVA and SNK

[e]$P < 0.05$ versus 2I,2R, both 3I,2R, and No Tr groups by ANOVA and SNK

Postexposure measurements

At completion of hyperoxia, one rat was removed from the chamber for data collection while the others remained in hyperoxia to wait their turn. The procedure for data collection was as follows. The rat was weighed and colonic body temperature was recorded as before. The rat was then sedated using isoflurane (Minrad Inc., Buffalo, NY, USA). Heparinized blood was collected from the abdominal aorta for hematocrit and plasma protein determinations (duplicate capillary tube blood samples were centrifuged at 11,000 rpm/5 min) and plasma protein values were obtained from the plasma portion of the hematocrit sample using a hand-held refractometer (Atago, Tokyo, Japan) with distilled water as a zero reference and values were standardized against albumin. Following exsanguination, the trachea was cannulated and the chest was opened by midline incision, with care taken to avoid blood vessels. Pleural effusion volume was recorded and effusion protein determination was made using the hand-held refractometer. The left lung was then ligated for determination of wet lung weight. Dry lung weights were obtained by heating the middle left lung lobe at 55°C until stable values were measured.

Statistical analysis

All data are expressed as means $\pm$ SEM with n equal to the number of animals per sample group. A one-way analysis of variance statistical program was used in association with the Student–Newman–Keuls test (Primer of Biostatistics: The Program, Stanton A Glantz, Version 3.0. McGraw-Hill, 1992) to determine statistical significance ($P < 0.05$) between comparable values for all groups and a t-test was used to determine statistical significance between two groups of animals.

Results

Zymosan priming and protection against oxygen toxicity

Rats develop proteinaceous pleural effusions and hemorrhagic lungs as indicators of pulmonary oxygen toxicity. Measurement of pleural effusion volume and effusion protein content and calculation of the ratio of effusion protein to plasma protein values therefore provide useful indices of hyperoxic lung injury. Additionally, wet/dry lung weight ratios indicate fluid accumulation in hyperoxic lungs; increasing hematocrit values (hemoconcentration) indicate a loss of plasma (lung water) from leaky pulmonary vessels; and lung appearance (LA) values (assigned after visual examination of the lung surface with 1 being normal in appearance and 9 being 'liver like') indicate the extent of surface hemorrhage. Taken together, these measurements provide a clear picture of the response of pulmonary tissues to hyperoxia and allow determinations to be made regarding protection by zymosan priming.

Study 1, role of dosage (Table 1)

Values in Z-1 rats for pleural effusion volume, effusion protein content, effusion protein/plasma protein (EP/PP) ratio, wet/dry (W/D) lung weight ratios, hematocrit (Hct) and LA were all significantly less than comparable values in Saline rats, demonstrating that 1 mg zymosan protects rats against pulmonary oxygen toxicity. The values for each measured parameter across the dosage range of 1–15 mg zymosan/injection for groups in hyperoxia (Z-1 to Z-15 groups) did not differ statistically but mean values tended to decrease with dosage, suggesting that zymosan protection increased with dosage.

Study 2, duration of rest (Table 2)

Effusion volume, EP/PP ratios, W/D lung weight ratios, hematocrit values and LA show that a one-day rest after injections provides partial protection against hyperoxia. In contrast, effusion protein levels were not statistically different from the Saline group values until rats received two days' rest (3I,2R group, Table 2). This demonstrates that full protection of rats requires two days' rest before hyperoxia.

Study 3, number of injections (Table 3)

One zymosan injection was sufficient to cause partial protection in terms of effusion volume and hematocrit (1I,2R group) and two injections caused EP/PP and W/D ratios to decrease significantly (2I,2R group) compared with Saline values. Significant decreases in all six measured indices of lung injury were not achieved until rats received three injections (3I,2R group), showing that a minimum of three injections were necessary to produce full protection against hyperoxia. As before, protection tended to increase as the number of injections increased.

Additional observations

Similar to endotoxin, zymosan-treated rats became lethargic and hyperthermic but, unlike endotoxin, zymosan treatment did not cause diarrhea. Colonic body temperatures peaked at 2–3 h in all zymosan-treated rats; those receiving 12 or 15 mg zymosan/injection ($n = 24$) developed fevers of 1.54 ± 0.11°C while body temperatures in rats receiving saline ($n = 3$) increased by 0.47 ± 0.33°C ($P = 0.004$, t-test). Although saline-treated rats did develop slight fevers (probably as a result of handling), they did not become lethargic.

Discussion

Proteinaceous pleural effusions are the hallmark of oxygen toxicity in the rat. Treatment with endotoxin has been a classic model to study protection of rats against oxygen toxicity, and the general consensus is that endotoxin protects rats by induction of antioxidant enzymes.[7]

We have previously reported that zymosan priming protects rats against oxygen toxicity.[9] In that study, rats received zymosan priming with endotoxin-free zymosan according to a standard priming protocol involving three daily intravenous injections with zymosan followed by two days' rest before hyperoxia.[3] This study was conducted to determine the limits of treatment parameters on zymosan priming protection.

We found that treatment of rats according to the standard priming protocol afforded the most complete protection against hyperoxia. Accordingly, changes from the standard priming protocol in terms of reduction of dosage, decrease in length of rest period or reduction in number of injections all caused an increase in measured values of lung injury (Tables 1–3). These observations are significant and suggest that the mechanism(s) of protection by zymosan priming against oxygen toxicity may involve cytokines and share similarities with other studies where the priming protocol was applied.[1–3] As such, the zymosan priming model may provide a new tool for oxygen toxicity research or studies in areas involving Toll-like receptors or adjuvancy.

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