Journal of Toxicology and Environmental Health, Part A

ISSN: 1528-7394 (Print) 1087-2620 (Online) Journal homepage: http://www.tandfonline.com/loi/uteh20

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To cite this article: Shih-Houng Young , Gary R. Ostroff , Patti C. Zeidler-Erdely , Jenny R. Roberts , James M. Antonini & Vincent Castranova (2007) A Comparison of the Pulmonary Inflammatory Potential of Different Components of Yeast Cell Wall , Journal of Toxicology and Environmental Health, Part A, 70:13, 1116-1124, DOI: 10.1080/15287390701212224

To link to this article: https://doi.org/10.1080/15287390701212224

Journal of Toxicology and Environmental Health, Part A, 70: 1116–1124, 2007

ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287390701212224

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A Comparison of the Pulmonary Inflammatory Potential of Different Components of Yeast Cell Wall

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1→3-β-Glucan has been associated with pulmonary inflammation induced by exposure to fungal or yeast cell wall dust. 1→3-β-Glucan is the major cell wall component of yeast or fungi. However, the yeast cell wall contains several other components besides 1→3-β-glucans, such as mannan and chitin. Few studies evaluated the contribution of these other cell wall components to pulmonary inflammation. The present study compares a crude particulate yeast cell wall preparation (zymosan A) to purified yeast glucan, purified yeast glucan mannan, or purified yeast glucan chitin particles for their potency to induce mouse pulmonary inflammation after in vivo exposure. Mannan is the second most abundant polysaccharide in the yeast cell wall, whereas chitin content is a minor component. The results show that pulmonary injury is mediated by both chitin and 1→3-β-glucan and to a lesser degree by mannan. There is also evidence that zymosan is more potent than purified 1→3-β-glucan alone. Evidence indicates that 1→3-β-glucan is the major inflammatory component in yeast and fungal cell walls.

1→3-β-Glucan is an important biomarker for detecting the presence of fungi or yeast. Furthermore, 1→3-β-glucan is also an important pulmonary inflammation marker of fungal exposure, regardless whether it is derived from viable or nonviable fungi. Therefore, measurement of 1→3-β-glucan is associated with exposure to fungi or yeast (Rylander, 1999). However, questions remain as to whether 1→3-β-glucan is the only agent associated with the fungal cell wall that can contribute to the observed inflammatory pulmonary responses (Ormstad et al., 2000). There is limited information in regard to the inflammatory potential of other major components of yeast cell wall (Domer & Garner, 1990; Limper et al., 2003; Schuyler et al., 1998; Sorenson et al., 1998). The main goal of the present investigation was to compare the inflammatory potential of each major cell wall component from yeast in a mouse model.

The main components of yeast cell wall are glucan, mannan, and chitin, which comprise 90% of the cell wall (Ballou, 1982). Fungal cell walls typically are 80% polysaccharide and 3–20% protein by dry weight (Ruiz-Herrera, 1991). The composition of each component may change, depending on the strain, growth stage, environmental conditions, and the presence of specific inhibitors (Ruiz-Herrera, 1991). For example, the composition of chitin in the cell wall may vary from 0 to 36%, depending on the stage of growth (Ruiz-Herrera, 1991). Zymosan A is a commercially available yeast *Saccharomyces cerevisiae* cell wall preparation. The composition of Zymosan A was reported to be about 70–75% total polysaccharide, ~13–17% protein, 0.8% chitin, 3–3.5% ash, and 6–7% fat (DiCarlo and Fiore 1957). Among the total polysaccharides, there are 50–57% glucans and 16–22% mannan. Thus, mannan is the second most abundant polysaccharide in yeast cell wall. Mannan is the generic name given to the polysaccharide moiety of glycoproteins (Ruiz-Herrera, 1991). Mannans are formed by a chain of 1→4-β-mannose (Heredia et al., 1995). Mannan is assumed to be the major antigen of *Candida albicans* and/or other *Candida spp*. cell walls (Domer & Garner, 1990).

Received 29 June 2006; accepted 14 August 2006.

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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Chitin is an unbranched polysaccharide composed of *N*-acetylglucosamine linked through 1→4-β bonds (Ruiz-Herrera, 1991). 1→3-β-Glucan, mannan, and chitin are linked together to form the rigid yeast or fungal cell wall. Smits et al. (1999) described a structural model of the cell wall. The internal skeletal framework is formed by a three-dimensional network of 1→3-β-glucan. The skeletal framework is strengthened by the crosslinked 1→3-β-glucan chitin chains. The linkage between chitin and 1→3-β-glucan was found by Kollar et al. (1995). Mature 1→6-β-glucan is mainly found at the outside of the skeletal framework (Kollar et al., 1995, 1997; Smits et al., 1999). These polysaccharides possess a structural function for fungal cell wall, whereas the mannoproteins may act as "filler" and are responsible for permeability of the cell wall (Kollar et al., 1997). Fungal cell walls are extremely rigid and resistant to enzymatic attack; such resistance is a prerequisite for fungal survival in a hostile environment (Ruiz-Herrera, 1991).

In the present study, mice were exposed by pharyngeal aspiration to either a crude preparation of yeast cell wall (zymosan A), or yeast cell wall preparations enriched in the mannan, chitin, or 1→3-β-glucan constituents. Pulmonary inflammation and injury parameters were measured 1 d after exposure in an attempt to determine which component of yeast cell wall was predominantly responsible for the effects observed.

MATERIALS AND METHODS

Animals and Pulmonary Treatment

Specific-pathogen-free male mice of strain C3HeB/FeJ (from Jackson Lab, Bar Harbor, ME) were used in the experiments. Mice were treated according to an Animal Care and Use Committee-approved in-house animal protocol. The animals were housed in a room with restricted access and HEPA-filtered air and were allowed to acclimate in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved animal facility for 1 wk before use. The mice were maintained on Harlan NIH-31, 6% irradiated diet and tap water ad libitum. Beta-chips were used as bedding. Mice (20–25 g) were exposed by pharyngeal aspiration to 40 μl of vehicle (sterile saline) or suspension of zymosan A, yeast glucan, yeast glucan mannan, or yeast glucan chitin particles at a concentration of 1.4 mg/ml, which is equal to approximately 2.5 mg/kg body weight. The pharyngeal aspiration method was according to Rao et al. (2003). Briefly, each mouse was anesthetized with isoflurane (Abbott laboratories, North Chicago, IL). When fully anesthetized, the mouse was placed on a slant board with the tongue pulled aside by small forceps. Then 40 μl of suspension was pipetted at the base of the tongue and tongue restraint was continued until at least 2 deep breaths were completed. This pharyngeal aspiration technique was shown to provide a more even distribution of particles in the lung than intratracheal instillation (Rao et al., 2003). Mice were euthanized at 1 d postexposure, as this time point was shown to exhibit a good inflammatory response (Young et al., 2001a), and bronchoalveolar lavage (BAL) was conducted.

Experimental Design

A dose-response curve for zymosan was generated in a preliminary experiment. It was determined that 2.5 mg/kg of body weight was the optimal dose to induce significant lung injury and inflammation in mice (Table 1). Therefore, a dose of 2.5 mg/kg body weight was used for all test samples. In order to compare the effects of glucan, mannan and chitin particles, two concentrations of yeast 1→3-β-glucan particles (YG1 and YG2) were used to adjust for the difference in glucan content in each cell wall component as shown in Table 2.

Chemicals

Yeast glucan particles (YG) purified from *Saccharomyces cerevisiae*, contained ~80% 1→6-β branched, 1→3-β-glucan, 2–4% chitin, and <1% mannan w/w. Yeast glucan mannan particles (YGM) purified from *Saccharomyces cerevisiae* contained ~40% 1→6-β branched, 1→3-β-glucan, 2–4% chitin, and ~40% mannan, w/w. Yeast chitin particles (YCP) purified from *Rhodotorula mucilaginosa* contained ~40–50% chitin

TABLE 1
Dose-Dependent Pulmonary Injury 1 d Postexposure to Zymosan

Chemical	LDH (U/L)	Albumin (mg/ml)
Saline	99.17 ± 4.64	0.11 ± 0.01
Zymosan (0.1 mg/kg)	108.17 ± 3.63	0.14 ± 0.01
Zymosan (1 mg/kg)	164.50 ± 3.69*	0.25 ± 0.02*
Zymosan (2.5 mg/kg)	232.60 ± 10.19*	0.35 ± 0.03*
Zymosan (5 mg/kg)	272.00 ± 30.25*	0.44 ± 0.09*

Note. Values are means + SEM (n = 6). Asterisk indicates significantly greater than mean value of saline controls ($p < .05$).

TABLE 2
Experimental Design for Correcting the Difference of β-Glucan Content in Yeast Glucan, Mannan, and Chitin

Chemical	Glucan	Mannan	Chitin
YG 2 (2.5 mg/kg)	80%	<1%	2–4%
YG 1 (1.25 mg/kg)	40%	<0.5%	1–2%
YGM (2.5 mg/kg)	40%	40%	2–4%
Chitin (2.5 mg/kg)	40–50%	0	40–50%

Note. YG, yeast glucan; YGM, yeast glucan mannan; chitin, yeast glucan chitin.

and ~40–50% 1→6-β branched, 1→3-β-glucan, and no mannan. The composition analysis of YG, YGM, and YCP is described in another section of this article. Zymosan was purchased from Sigma (St. Louis, MO). Concanavalin A (ConA)-594 was purchased from Molecular Probes (Alexa Fluor 594, catalogue number C-11253, Eugene, OR). All cell-wall components were tested for endotoxin content and β-glucan content.

Fluorescent Microscopic Evaluation of Yeast Glucan Particles (YG), Yeast Glucan Mannan Particles (YGM), and Zymosan by ConA-594 Staining and Particle Number Counting

For concanavalin A (ConA)-594 staining, particle concentration was adjusted to 1×10^7/ml in PBS + 1 m*M* $MgCl_2$ and 1 m*M* $CaCl_2$. The particles were incubated with 10 μg ConA-594 for 1 h at room temperature. ConA selectively binds to α-mannopyranosyl and α-glucopyranosyl residues. The particle samples were washed with PBS 3 times by centrifugation at 10,000 × g for 5 min, resuspended in phosphate-buffered saline (PBS) + 1% formalin, and imaged by light and fluorescence microscopy at 400×. The particle number for each chemical used was determined at a concentration of 1.4 or 0.7 mg/ml, with a Coulter electronic multisizer II (Coulter Electronics, Hialeah, FL). The results are shown in Table 3.

Yeast Glucan, Mannan, and Chitin Composition Analysis

The yeast composition was determined using the method of Dallies et al. (1998). The carbohydrate composition of acid-hydrolyzed yeast cell wall preparations was determined by Dionex high-performance liquid chromatography (HPLC) using pulsed amphometric detection. For cell-wall hydrolysis, yeast cell walls (1 mg) were incubated with 100 μl 72% (w/w) H_2SO_4 and incubated at room temperature for 3 h. The sample was diluted to 2 *N* H_2SO_4 by the addition of 900 μl MilliQ water and heated in sealed tubes for 4 h at 100°C. After cooling, the hydrolysate was diluted with 9 ml MilliQ water. Sulfate ions were precipitated by the addition of saturated $Ba(OH)_2$ to neutral pH. The volume was adjusted to 25 ml and the $BaSO_4$ precipitate was removed by centrifugation. Quantification of monosaccharides by high-performance anion-exchange chromatography (HPAEC) of samples was performed using a Dionex Bio-LC system (Sunnyvale, CA). Monosaccharides liberated by acid hydrolysis (glucose, mannose, and glucosamine) were separated on a Dionex CarboPac PA1 anion-exchange column. Isocratic elution was performed at room temperature with 18 m*M* NaOH at a flow rate of 1 ml/min for 30 min. Detection of sugars was performed by a pulsed amperometric detection (PAD, Dionex) using a gold electrode. Quantification of sugars was performed using the response factors calculated from the peak areas of reference monosaccharides.

TABLE 3
Particle Counts for Each of Cell Wall Components (Corrected for Saline Background Counting)

Chemical	Particle counts (10^6)
Saline	0
Zymosan (1.4 mg/ml)	48.64
YGM (1.4 mg/ml)	22.87
YG 1 (0.7 mg/ml)	38.75
YG 2 (1.4 mg/ml)	83.75
Chitin (1.4 mg/ml)	16.42

Note. YG, yeast glucan; YGM, yeast glucan mannan; chitin, yeast glucan chitin.

Endotoxin Test

The endotoxin test was conducted using a kinetic QCL procedure (BioWhittaker, Walkersville, MD, catalogue number 50–650U). Briefly, all the test samples were prepared in a PBS suspension at the indicated concentration. Aliquots of 100 μl of endotoxin standard, blank, or sample were added to each well. Then 100 μl of *Limulus* amebocyte lysate (LAL) were added to each well and the OD was monitored at 405 nm for 40 min. The time of onset (OD = 0.2) was measured, and the corresponding endotoxin content was calculated for each sample. The results indicated that the endotoxin content of the test samples was 0.3–29 EU/ml, which was reported in our earlier studies to be too low to induce any significant inflammation in rats (Young et al., 2002).

β-Glucan Assay

The specific 1→3-β-glucan content in each sample was analyzed with a Glucatell kit (Associates of Cape Cod, Falmouth, MA). The onset time was set at OD = 0.03. The detecting wavelength was set at 405 nm, measured every 20 s, and was continued for 1 h. For a concentration of 1.4 mg/ml of each sample, Glucatell detected glucan content as follows: zymosan 0.215 μg/ml, yeast glucan 0.32 μg/ml, yeast glucan mannan 0.18 μg/ml, and yeast glucan chitin 24.28 μg/ml. The Glucatell results were not consistent with the composition analysis results. This may be due to the fact that Glucatell detects extractable or accessible 1→3-β-glucan from suspension, and particulate 1→3-β-glucans used in this study were insoluble in water. Therefore, only a relative small fraction of total 1→3-β-glucan can be detected using Glucatell.

Bronchoalveolar Lavage and Cell Differentials

At d 1 postexposure, the mice were deeply anesthetized with an overdose of Sleepaway (>100 mg/kg sodium pentobarbital/mouse, Fort Dodge Animal Health, Fort Dodge, IA) and then exsanguinated by severing the abdominal aorta. Their lungs were first lavaged with 0.6 ml of Ca^{2+}- and Mg^{2+}-free cold phosphate-buffered solution (PBS) at pH 7.4. The first

fraction of lavage fluid was retained in the lungs for 30 s with constant massaging of the lungs until collection. This first fraction of BAL fluid was centrifuged at 500 × g for 10 min, and the supernatant was used for analyzing lactate dehydrogenase (LDH) activity and albumin and cytokines levels. The lungs were further lavaged with 1-ml aliquots of PBS until a total of 5 ml BAL fluid was collected. These samples were also centrifuged for 10 min at 500 × g, and the cell pellets from all washes for each mouse were combined and used for cell differentials. Total cell number was determined with a Coulter electronic multisizer II (Coulter Electronics, Hialeah, FL). For cell differentiation, 5×10^5 cells were spun for 5 min at 72 × g and pelleted onto a slide using a cytospin (Shandon Cytospin II, Shandon, Inc., Pittsburgh, PA). Cells (200 cells/mouse) were counted and identified as alveolar macrophages, lymphocytes, eosinophils, or neutrophils on cytocentrifuge-prepared slides after staining with Leukostat stain (Fisher Scientific, Pittsburgh PA).

Biochemical Parameters of Injury

The albumin content and LDH activity in the acellular first fraction of BAL fluid were measured. These measures reflect the permeability of the bronchoalveolar–capillary barrier and general cytotoxicity, respectively. Albumin content was determined colorimetrically at 628 nm based on albumin binding to bromcresol green using an albumin BCG diagnostic kit (Sigma, St. Louis, MO). LDH activity was determined by measuring the reduction of lactate to pyruvate coupled with the formation of NADH at 340 nm. Measurement was performed with a COBAS MIRA autoanalyzer (Roche Diagnostic System, Montclair, NJ).

BAL Cytokines

Analysis of cytokines from the acelluar BAL fluid was conducted using a mouse inflammation cytometric bead array kit (BD Biosciences, San Diego, CA) and was analyzed on a FACSCalibur flow cytometer. Standard curves were determined for each cytokine in a range of 20–5000 pg/mL. The lower detect limit of assay is 2.5–52.7 pg/ml, depending on types of cytokines. The following cytokines were measured: interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), interferon- γ (IFN-γ), tumor necosis factor-α (TNF-α), and interleukin-12p70 (IL-12p70).

Statistical Analysis

All data are presented as means ± standard error of mean (SEM). Statistical analysis was performed using SigmaStat v3.11 software (Systat Software, Inc.). The significance was set at $p \leq .05$. For multiple comparisons, a one-way analysis of variance (ANOVA) with Student–Newman–Keuls procedure was used for comparing several treatment groups with one control and making a comparison between each treatment groups.

RESULTS

Fluorescence Microscopy

The light and fluorescence photomicrographs are shown in Figure 1. YG, YGM, and zymosan morphologically resemble yeast cell wall ghosts. YG do not contain mannan as evidenced by the absence of ConA-594 fluorescence (Figure 1, A and B). ConA-594 avidly binds to YGM as evidenced by bright marginal fluorescence (Figure 1, C and D). ConA-594 also binds avidly to zymosan as confirmed by bright marginal fluorescence (Figure 1, E and F), which suggests that zymosan contains mannan on its surface.

Pulmonary Inflammation, Cytotoxicity, and Lung Injury

LDH Activity and Albumin Content

Pulmonary cell damage resulting from exposure to the components of yeast cell wall was evaluated by measuring LDH activity and albumin levels leaked in the acellular first fraction of BAL fluid. Table 1 shows a dose-dependent increase in pulmonary injury 1 d after zymosan exposure. A statistically significant elevation was observed in both LDH activity and albumin levels at 1, 2.5, and 5 mg/kg zymosan compared to control.

Figure 2 shows LDH activities from the first fraction of BAL fluid after treatment with different cell wall components. All test cell wall components induced significantly higher levels of LDH activity than in the control group. Two doses of yeast glucan induced a dose-dependent increase in LDH activity. Zymosan, the crude yeast cell wall preparation, induced the highest level of LDH activity.

Potency Analysis. In order to compare the relative cytotoxicity (LDH) between treatment with mannan, 1→3-β-glucan (YG1), and chitin alone, the LDH value in response to YG1 (containing a similar levels of β-glucan as the mannan or chitin sample) was subtracted from the experimental LDH value of mannan and chitin. The cytotocixity produced by YG1 alone was calculated by subtracting the LDH value of the saline group from that of YG1 group. The results suggest that the relative cytotoxicity of the samples was ranked as chitin > glucan > mannan. The same process of potency analysis was conducted for other endpoints.

Figure 3 shows the albumin levels in the first fraction of BAL fluid from the different treatment groups. Using the potency analysis procedure for correcting for β-glucan in the mannan and chitin which was described in the LDH section, the relative order of pulmonary injury as assessed by measuring albumin levels was chitin > glucan > mannan.

Total BAL Cell

Figure 4 shows the total nucleated cell count from the recovered BAL fluid after treatment. Zymosan treatment produced the greatest infiltration of inflammatory cells into the lungs compared to other yeast cell wall component. Two

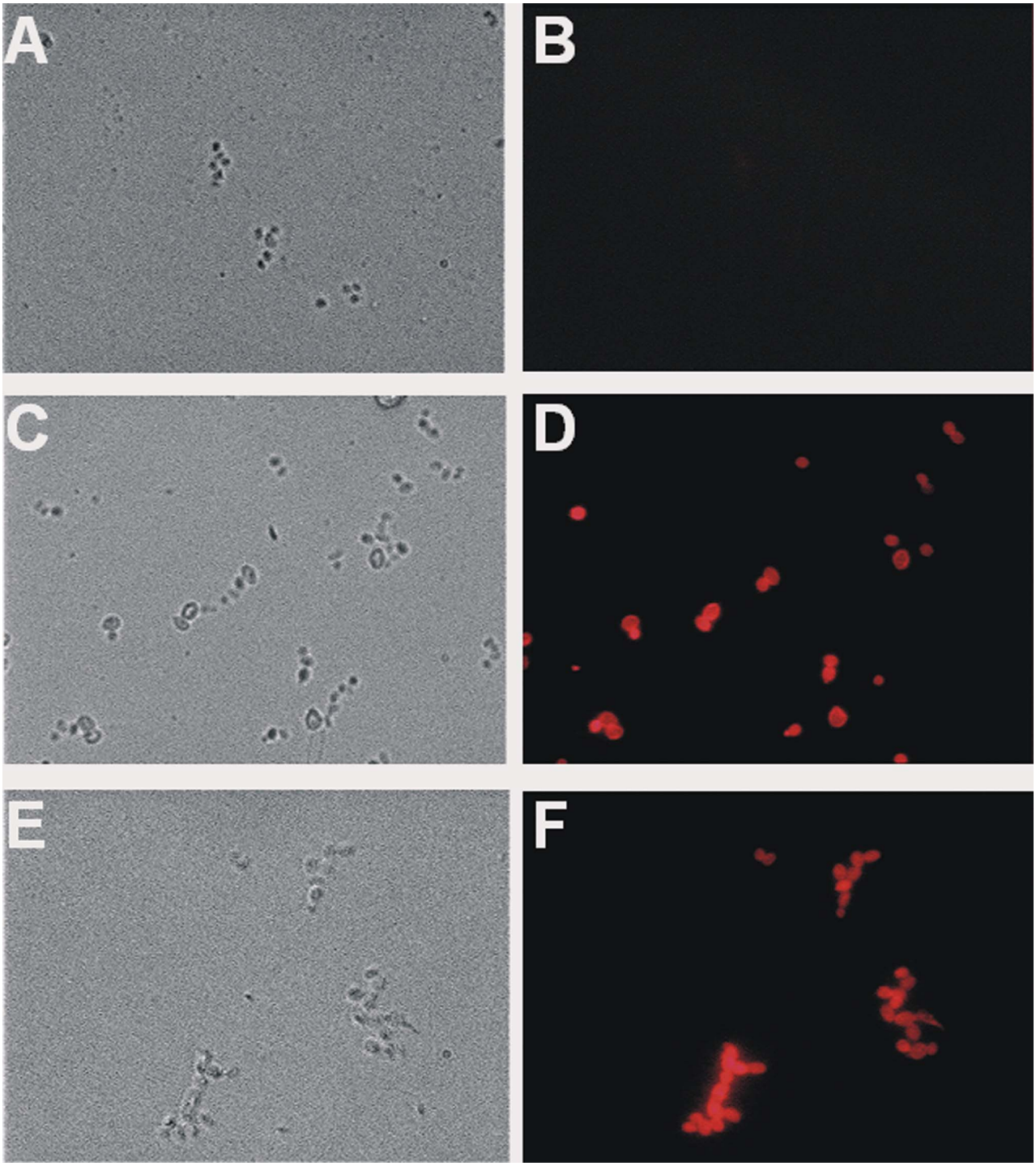

FIG. 1. (A and B) Yeast glucan particles, ConA-Texas red stained; (A) is under regular white light and (B) is the fluorescence image. (C and D) Yeast glucan mannan particles, ConA-Texas red stained; (C), white light and (D) fluorescence. (E and F) Zymosan particles, ConA-Texas red stained; (E) white light and (F) fluorescence.

doses of yeast glucan induced a dose-dependent increase in total BAL cells. The order of potency, corrected for the β-glucan in the mannan and chitin sample, was glucan > mannan > chitin.

BALF PMNs

Figure 5 shows the infiltration of polymorphonuclear neutrophils (PMN) from the different treatment groups. Zymosan induced a statistically significant higher PMN infiltration than

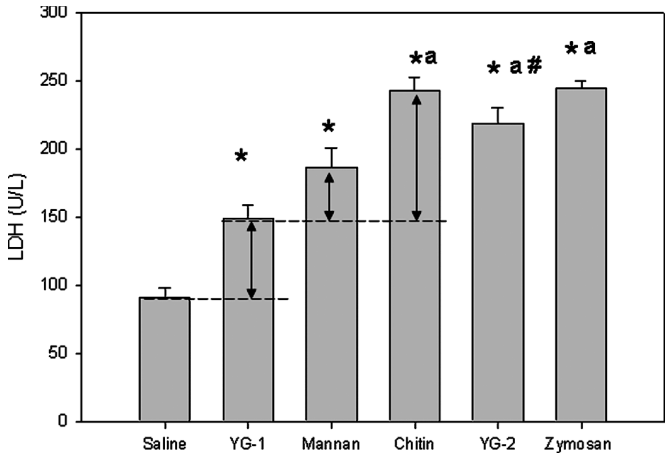

FIG. 2. Lactate dehydrogenase (LDH) activity in BAL fluid 1 d postexposure. Values are means ± SEM of eight mice per exposure group. Asterisk indicates a significant increase ($p < .05$) versus the control level; a, significantly higher than mannan; #, YG2 is significantly higher than YG-1. Dashed line denotes glucan or saline baseline level in the samples. Corrected potency ranking, chitin > glucan > mannan.

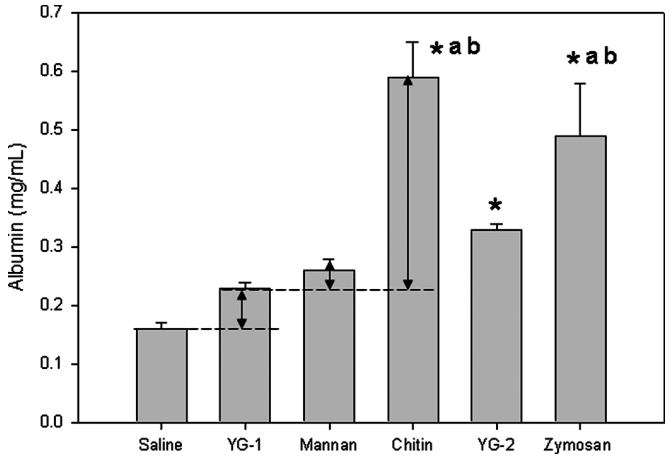

FIG. 3. Albumin concentrations in BAL fluid 1 d postexposure. Values are means ± SEM of eight mice per exposure group. Asterisk indicates a significant increase ($p \leq .05$) versus the control level; a, significantly higher than mannan; b, significantly higher than YG2. Dashed line denotes glucan or saline baseline level in the samples. Corrected potency ranking, chitin > glucan > mannan.

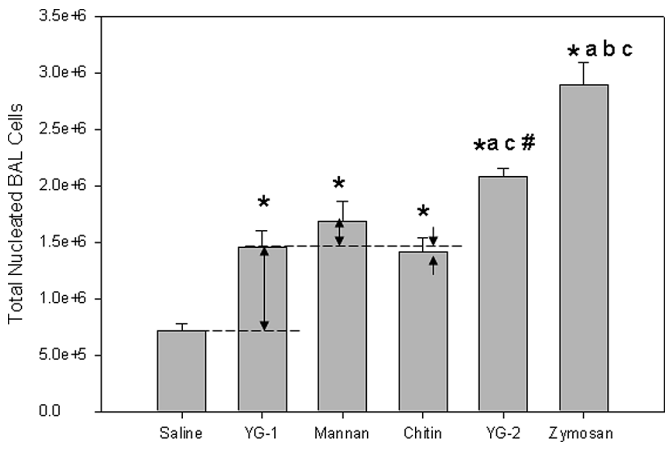

FIG. 4. Total nucleated BAL cell counts. Values are means ± SEM of eight mice per exposure group. Asterisk indicates a significant increase ($p < .05$) versus the control level; a, significantly higher than mannan; b, significantly higher than YG2; c, significantly higher than chitin; #, YG2 is significantly higher than YG-1. Dashed line denotes glucan or saline baseline level in the samples. Corrected potency ranking, glucan > mannan > chitin.

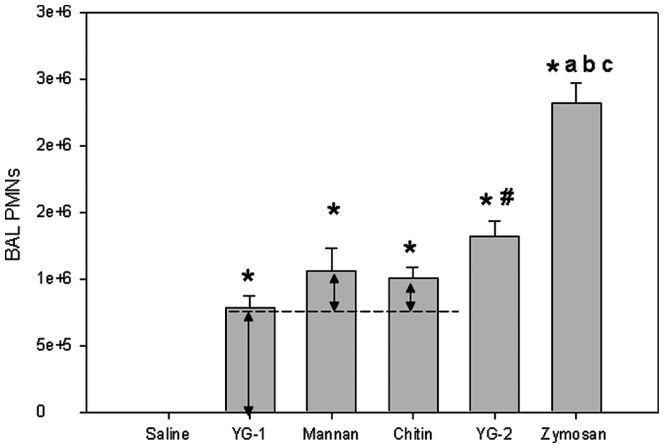

FIG. 5. Total BAL PMN counts. Values are means ± SEM of eight mice per exposure group. Asterisk indicates a significant increase ($p < .05$) versus the control level; a, significantly higher than mannan; b, significantly higher than YG2; c, significantly higher than chitin; #, YG2 is significantly higher than YG-1. Dashed line denotes glucan or saline baseline level in the samples. Corrected potency ranking, glucan > mannan = chitin.

the rest of the treatment groups. PMN infiltration was shown to have a dose-response relationship with zymosan (Young et al., 2001a). A similar dose-response was observed in YG1 and YG2. Glucan is the major component in zymosan. The order of potency, corrected for the β-glucan in the mannan and chitin sample, was glucan > mannan=chitin.

BALF Cytokines

The first fraction of BAL fluid from each mouse was analyzed for IL-6, IL-10, IL12p70, TNF-α, IFN-γ, and MCP-1 content. IL-10, IL12p70, and IFN-γ were not elevated in any of the treatment groups compared to the saline control group (data not shown). However, differences in expressions of TNF-α, MCP-1 and IL-6 cytokines were detected among the groups (Figures 6–8). Figure 6 shows that TNF-α was elevated in all the treatment groups compared to saline control. Zymosan-induced TNF-α expression was significantly higher than all other treatments. There was no dose-response relationship in two doses of yeast glucan. The reason for this was unclear and

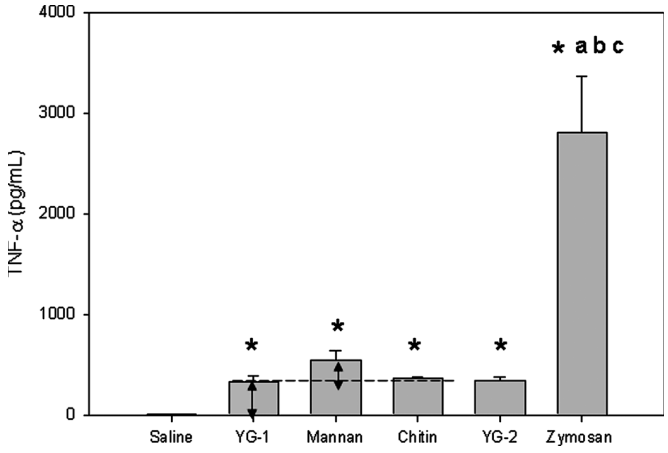

FIG. 6. BAL fluid TNF-α concentration (pg/ml). Values are means ± SEM of eight mice per exposure group. Asterisk indicates a significant increase ($p < .05$) versus the control level; a, significantly higher than mannan; b, significantly higher than YG2; c, significantly higher than chitin. Dashed line denotes glucan or saline baseline level in the samples. The corrected potency is glucan > mannan > chitin.

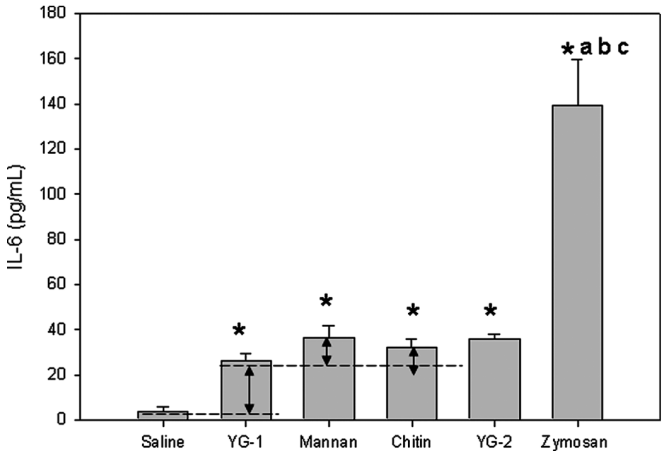

FIG. 8. BAL fluid IL-6 concentration (pg/ml). Values are means ± SEM of eight mice per exposure group. Asterisk indicates a significant increase ($p < .05$) versus the control level; a, significantly higher than mannan; b, significantly higher than YG2; c, significantly higher than chitin. Dashed line denotes glucan or saline baseline level in the samples. The corrected potency is glucan > mannan > chitin.

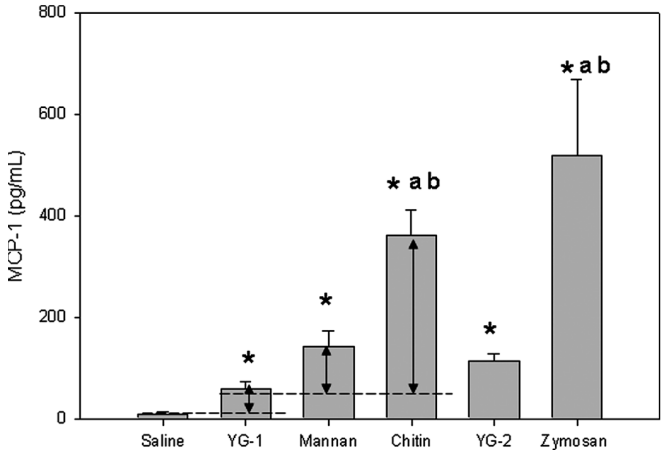

FIG. 7. BAL fluid MCP-1 concentration (pg/ml). Values are means ± SEM of 8 mice per exposure group. Asterisk indicates a significant increase ($p < .05$) versus the control level; a, significantly higher than mannan; b, significantly higher than YG2. Dashed line denotes glucan or saline baseline level in the samples. The corrected potency is chitin > mannan > glucan.

may reflect maximal stimulation by these doses of yeast glucan. The corrected potency was glucan > mannan > chitin. Figure 7 shows that the MCP-1 levels were significantly higher than saline control for all treatments. Zymosan- and chitin-induced MCP-1 expression were significantly higher than other treatments but were not different from each other. The corrected potency was chitin > mannan > glucan. Figure 8 shows that the IL-6 levels were significantly higher than saline control from all treatments. In addition, zymosan treatment induced a significantly elevated IL-6 response compared to other treatments. The corrected potency was glucan > mannan > chitin.

SUMMARY AND DISCUSSION

It is well known that exposure to fungal cell wall components induced pulmonary inflammation (Schuyler et al., 1998). Since 1→3-β-glucan is the major cell wall component, it was hypothesized that 1→3-β-glucan is the main component that induces pulmonary inflammation. Because of a lack of evidence from the scientific literature, it is not clear whether other components also contribute to pulmonary inflammation. In our previous studies, data demonstrated that exposure to zymosan A, a particulate form of 1→3-β-glucan from *Saccharomyces cerevisiae*, induced pulmonary damage and inflammation in rats (Young & Castranova, 2005; Young et al., 2002, 2003a, 2003b). In the present study, the inflammatory potential of a crude yeast preparation (zymosan) and semipurified particulate yeast cell wall components, including enriched yeast cell wall preparations of glucan–mannan, glucan–chitin, and purified yeast 1→3-β-glucan, was compared. Mannan is the major antigen of *Candida albicans* and/or other *Candida* spp. walls (Domer & Garner, 1990). Chitin is part of the rigid structure that forms the cell wall. The inflammatory potential of yeast chitin has not been adequately studied. It is important to note that the soluble part of the cell wall components was not under investigation in the current study, because previously it was shown that the particulate component of the cell wall is much more inflammatory than the soluble component (Young et al., 2003b).

1→3-β-Glucan is the main component in zymosan (DiCarlo & Fiore, 1957) and was shown to exert a dose-dependent effect on

cytotoxicity and damage to the blood barrier in animals. Data from the present investigation indicate that the relative cytotoxicity of the cell wall components can be ranked as chitin > glucan > mannan. For inflammation, the rank is glucan > mannan ≥ chitin. Together, these facts suggest that 1→3-β-glucan plays an important role in the recruitment of inflammatory cells into the lungs and resultant damage after exposure to zymosan. In this experiment, zymosan exhibits the greatest inflammatory potential compared to either glucan or chitin or mannan. This may due to the fact that zymosan contains all three components (glucan, mannan, and chitin), while others only have two components. Further, zymosan represents a more natural presentation of these yeast wall components, especially in the outer wall that is interacting with cells in the lung. Yeast glucan (YG) and yeast chitin glucan particles have had their outer wall mannan removed by alkaline degradation. This exposes the underlying glucan and chitin. In contrast, yeast mannan glucan (YGM) have only had some of their mannan removed by mild alkaline washing. Zymosan represents the most intact mannan structure.

The results from the commercially available 1→3-β-glucan specific assay do not correlate well with the inflammatory potential observed from this animal experiment. Although glucan-specific assay is a commonly used assay for detecting environmental glucan levels, the results from detection of 1→3-β-glucans with glucan-specific assay gave a much lower value than the results from the chemical composition analysis. This may be due to the fact that the 1→3-β-glucan in these cell wall preparations was insoluble in PBS. In addition, the particulate 1→3-β-glucan may not be accessible and may be poorly detected using the 1→3-β-glucan-specific assay. There is a need to develop a method to detect insoluble 1→3-β-glucan in samples, because the inflammatory response is greater to the insoluble component of 1→3-β-glucan (Young et al., 2003b).

Endotoxin is a potential confounding factor in pulmonary inflammation study. Endotoxin is a cell wall product from gram-negative bacteria and has been shown to be strongly associated with lung disease (Jacobs, 1989). The endotoxin assay results indicated that the endotoxin content of the test samples was 0.3–29 EU/ml, which was too low to induce any significant inflammation in rats from our earlier studies (Young et al., 2002). Furthermore, the endotoxin kit from Bio-Whittaker can cross react with 1→3-β-glucan (Cooper et al., 1997; Novitsky, 1993; Soderhall, 1983; Zhang et al., 1994), which resulted in a higher endotoxin readings. This suggests that the true endotoxin levels were even lower than the readings. There are methods to test the interference in LAL assay (Foto et al., 2004; Milton et al., 1990, 1992; Tsuchiya et al., 1990; Zhang et al., 1994). However, because of low endotoxin content in these samples, additional assays were not conducted. The interactions between endotoxin and 1→3-β-glucan was reported before (Young et al., 2002). There were no synergistic effects observed in animals treated with lipopolysaccharide (endotoxin) and 1→3-β-glucan. Instead, reduced pulmonary responses was observed when they were prereated with 1→3-β-glucan (zymosan A) then exposed to endotoxin (Young et al., 2002).

TNF-α is a proinflammatory cytokine released from activated macrophages and T lymphocytes in response to microbes or other agents. TNF-α plays a key role in the initiation of inflammation in the lungs and other tissues (Driscoll et al., 1997). Zymosan-induced TNF-α production occurs through the activation of nuclear factor κB (Young et al., 2001b). In the current study, it was observed that all treatments groups induced significantly higher TNF-α expression than saline control. The TNF-α response of zymosan was much higher than the other groups. Expression of IL-6, another proinflammatory cytokine, followed a similar pattern to that of TNF-α after treatment. These increased levels of TNF-α and IL-6 are consistent with a previous epidemiology study that reported an elevation of TNF-α, IL-6 and NOx in the nasal lavage fluid of school staff associated with exposure to mold microbes (Hirvonen et al., 1999). A similar rise in TNF-α and IL-6 was reported in the BAL fluid of mice exposed to fungal spore *Aspergillus versicolor* (Jussila et al., 2002).

MCP-1 is a chemoattract protein for monocytes. All treatments induced a significantly higher expression of MCP-1 as compared to saline control. Zymosan produced the greatest response compared to the other treatment groups. 1→3-β-Glucan was shown to activate macrophages (Burgaleta et al., 1978; Daum & Rohrbach, 1992; Di Luzio, 1979; Sakurai et al., 1995; Sherwood et al., 1986; Williams et al., 1996). The increased MCP-1 expression following treatment by yeast chitin–glucan particles versus mannan–glucan particles may be explained by the fact that chitin–glucan particles contain the highest extractable amount of 1→3-β-glucan compared to the other components as suggested by the glucan-specific assay. Cytokines IL-10, IFN-γ, and IL-12p70 were not elevated in BAL of any of the groups.

To further define the relative contributions of these yeast cell wall components, studies are underway to investigate the effect of antibodies administration to block their known receptors present on alveolar macrophages (chitin–mannose receptor [Han et al., 2005], 1→3-β-glucan–CR3 [Ross et al., 1987; Xia et al., 1999] and dectin-1[Brown et al., 2003], mannan–mannose receptor [Han et al., 2005; Turner, 2003], SIGNR1[Taylor et al., 2004], etc.).

In summary, the present investigation suggests that chitin and 1→3-β-glucan induce greater lung cytotoxicity and damage than mannan. Although chitin induced more blood–air barrier damage than 1→3-β-glucan or mannan, only a limited amount of chitin was present in the cell wall (0.8% chitin was detected in zymosan) (DiCarlo & Fiore, 1957). Therefore, 1→3-β-glucan is considered to be the major inflammatory component in fungal cell wall.

REFERENCES

Ballou, C. E. 1982. Yeast cell wall and cell surface. In T*he molecular biology of the yeast Saccharomyces, Metabolism and gene expression*, eds. J. N. Strathern, E. W. Jones, and J. R. Broach, pp. 335–306. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Brown, G. D., Herre, J., Williams, D. L., Willment, J. A., Marshall, A. S., and Gordon, S. 2003. Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med.* 197:1119–1124.

Burgaleta, C., Territo, M. C., Quan, S. G., and Golde, D. W. 1978. Glucan–activated macrophages: Functional characteristics and surface morphology. *J. Reticuloendothel. Soc.* 23:195–204.

Cooper, J. F., Weary, M. E., and Jordan, F. T. 1997. The impact of non-endotoxin lal-reactive materials on *Limulus* amebocyte lysate analyses. *PDA J. Pharm. Sci. Technol.* 51:2–6.

Dallies, N., Francois, J., and Paquet, V. 1998. A new method for quantitative determination of polysaccharides in the yeast cell wall. Application to the cell wall defective mutants of saccharomyces cerevisiae. *Yeast* 14:1297–1306.

Daum, T., and Rohrbach, M. S. 1992. Zymosan induces selective release of arachidonic acid from rabbit alveolar macrophage via stimulation of a beta-glucan receptor. *FEBS Lett.* 309, 119–122.

DiCarlo, F. J., and Fiore, J. V. 1957. On the composition of zymosan. *Science* 127:756–757.

Di Luzio, N. R. 1979. Lysozyme, glucan-activated macrophages and neoplsia. *J. Reticuloendothel. Soc.* 26:67–81.

Domer, J., and Garner, R. 1990. Fungal wall components and immunostimulation. In *Fungal cell wall and immune response*, J.-P. Latge and D. Boucias, pp. 415–430. Berlin: Springer-Verlag.

Driscoll, K. E., Carter, J. M., Hassenbein, D. G., and Howard, B. 1997. Cytokines and particle-induced inflammatory cell recruitment. *Environ. Health Perspect.* 105(suppl. 5):1159–1164.

Foto, M., Plett, J., Berghout, J., and Miller, J. D. 2004. Modification of the *Limulus* amebocyte lysate assay for the analysis of glucan in indoor environments. *Anal. Bioanal. Chem.* 379:156–162.

Han, Y., Zhao, L., Yu, Z., Feng, J., and Yu, Q. 2005. Role of mannose receptor in oligochitosan-mediated stimulation of macrophage function. *Int. Immunopharmacol.* 5:1533–1542.

Heredia, A., Jimenez, A., and Guillen, R. 1995. Composition of plant cell walls. *Z. Lebensm. Unters. Forsch.* 200:24–31.

Hirvonen, M. R., Ruotsalainen, M., Roponen, M., Hyvarinen, A., Husman, T., Kosma, V. M., Komulainen, H., Savolainen, K., and Nevalainen, A. 1999. Nitric oxide and proinflammatory cytokines in nasal lavage fluid associated with symptoms and exposure to moldy building microbes. *Am. J. Respir. Crit. Care Med.* 160:1943–1946.

Jacobs, R. R. 1989. Airborne endotoxins: An association with occupational lung disease. *Appl. Ind. Hyg.* 4:50–56.

Jussila, J., Komulainen, H., Kosma, V. M., Nevalainen, A., Pelkonen, J., and Hirvonen, M. R. 2002. Spores of aspergillus versicolor isolated from indoor air of a moisture-damaged building provoke acute inflammation in mouse lungs. *Inhal. Toxicol.* 14:1261–1277.

Kollar, R., Petrakova, E., Ashwell, G., Robbins, P. W., and Cabib, E. 1995. Architecture of the yeast cell wall. The linkage between chitin and beta(1-->3)-glucan. *J. Biol. Chem.* 270:1170–1178.

Kollar, R., Reinhold, B. B., Petrakova, E., Yeh, H. J., Ashwell, G., Drgonova, J., Kapteyn, J. C., Klis, F. M., and Cabib, E. 1997. Architecture of the yeast cell wall. Beta(1-->6)-glucan interconnects mannoprotein, beta(1-->)3-glucan, and chitin. *J. Biol. Chem.* 272:17762–17775.

Limper, A. H., Lebron, F., Evans, S. E., and Hahn, R. Y. 2003. *Pneumocystis carinii*: Cell wall beta-glucan-mediated pulmonary inflammation. *J. Eukaryot. Microbiol.* 50(suppl.):646.

Milton, D. K., Gere, R. J., Feldman, H. A., and Greaves, I. A. 1990. Endotoxin measurement: Aerosol sampling and application of a new limulus method. *Am. Ind. Hyg. Assoc. J.* 51:331–337.

Milton, D. K., Feldman, H. A., Neuberg, D. S., Bruckner, R. J., and Greaves, I. A. 1992. Environmental endotoxin measurement: The kinetic *Limulus* assay with resistant-parallel-line estimation. *Environ. Res.* 57:212–230.

Novitsky, T. J. 1993. Comparison of various LAL methods. *LAL Update* 11:2–4.

Ormstad, H., Groeng, E.-C., Lovik, M., and Hetland, G. 2000. The fungal cell wall component beta-1,3-glucan has an adjuvant effect on the allergic response to ovalbumin in mice. *J. Toxicol. Environ. Health A* 61:55–67.

Rao, G. V., Tinkle, S., Weissman, D. N., Antonini, J. M., Kashon, M. L., Salmen, R., Battelli, L. A., Willard, P. A., Hoover, M. D., and Hubbs, A. F. 2003. Efficacy of a technique for exposing the mouse lung to particles aspirated from the pharynx. *J. Toxicol. Environ. Health A* 66:1441–1452.

Ross, G. D., Cain, J. A., Myones, B. L., Newman, S. L., and Lachmann, P. J. 1987. Specificity of membrane complement receptor type three (cr3) for beta-glucans. *Complement* 4:61–74.

Ruiz-Herrera, J. 1991. *Fungal cell wall: Structure, synthesis, and assembly*. Ann Arbor, MI: CRC Press.

Rylander, R. 1999. Indoor air-related effects and airborne (1 --> 3)-beta-d-glucan. *Environ. Health Perspect.* 107(suppl. 3):501–503.

Sakurai, T., Ohno, N., Suzuki, I., and Yadomae, T. 1995. Effect of soluble fungi (1–3)-beta-d-glucan obtained from sclerotinia sclerotiorum on macrophage activation. *Immunopharmacology* 30:157–166.

Schuyler, M., Gott, K., and Cherne, A. 1998. Effect of glucan on murine lungs. *J. Toxicol. Environ. Health A* 53:493–505.

Sherwood, E. R., Williams, D. L., and Di Luzio, N. R. 1986. Glucan stimulates production of antitumor cytolytic/cytosatic factor(s) by macrophages. *J. Biol. Response Mod.* 5:504–526.

Smits, G. J., Kapteyn, J. C., van den Ende, H., and Klis, F. M. 1999. Cell wall dynamics in yeast. *Curr. Opin. Microbiol.* 2:348–352.

Soderhall, K. 1983. Beta-1,3-glucan enhancement of protease activity in crayfish hemocyte lysate. *Comp. Biochem. Physiol. B* 74:221–224.

Sorenson, W. G., Shahan, T. A., and Simpson, J. 1998. Cell wall preparations from environmental yeasts: Effect on alveolar macrophage function in vitro. *Ann. Agric. Environ. Med.* 5:65–71.

Taylor, P. R., Brown, G. D., Herre, J., Williams, D. L., Willment, J. A., and Gordon, S. 2004. The role of signr1 and the beta-glucan receptor (dectin-1) in the nonopsonic recognition of yeast by specific macrophages. *J. Immunol.* 172:1157–1162.

Tsuchiya, M., Oishi, H., Takaoka, A., Fusamoto, M., and Matsuura, S. 1990. Discrimination betweenn endotoxin and (1->3)-beta-d-glucan using turbidimetric kinetic assay with limulus amebocyte lysate. *Chem. Pharm. Bull.* 38(9):2523–2526.

Turner, M. W. 2003. The role of mannose-binding lectin in health and disease. *Mol. Immunol.* 40:423–429.

Williams, D. L., Mueller, A., and Browder, W. 1996. Glucan-based macrophage stimulators—A review of their anti-infective potential. *Clin. Immunother.* 5:392–399.

Xia, Y., Vetvicka, V., Yan, J., Hanikyrova, M., Mayadas, T., and Ross, G. D. 1999. The beta-glucan-binding lectin site of mouse cr3 (cd11b/cd18) and its function in generating a primed state of the receptor that mediates cytotoxic activation in response to ic3b-opsonized target cells. *J. Immunol.* 162:2281–2290.

Young, S.-H., and Castranova, V. 2005. Animal model of (1->3)-beta-glucan-induced pulmonary inflammation in rats. In *Toxicology of 1->3-beta-glucans, Glucans as a marker for fungal exposure*, eds. S.-H. Young and V. Castranova, pp. 65–93. New York: Taylor & Francis.

Young, S.-H., Robinson, V. A., Barger, M., Porter, D. W., Frazer, D. G., and Castranova, V. 2001a. Acute inflammation and recovery in rats after intratracheal instillation of a 1-> 3 -beta-glucan (zymosan a). *J. Toxicol. Environ. Health A* 64:311–325.

Young, S.-H., Ye, J., Frazer, D. G., Shi, X., and Castranova, V. 2001b. Molecular mechanism of TNF-a production in 1->3-b-glucan (zymosan) activated macrophages. *J. Biol. Chem.* 276:20781–20787.

Young, S.-H., Robinson, V. A., Barger, M., Zeidler, P., Porter, D. W., Frazer, D. G., and Castranova, V. 2002. Modified endotoxin responses in rats pre-treated with 1->3-beta -glucan (zymosan a). *Toxicol. Appl. Pharmacol.* 178:172–179.

Young, S.-H., Robinson, V. A., Barger, M., Frazer, D. G., Castranova, V., and Jacobs, R. R. 2003a. Partially opened triple helix is the biologically active conformation of 1-->3-beta-glucans that induces pulmonary inflammation in rats. *J. Toxicol. Environ. Health A* 66:551–563.

Young, S.-H., Robinson, V. A., Barger, M., Whitmer, M., Porter, D. W., Frazer, D. G., and Castranova, V. 2003b. Exposure to particulate 1-->3-beta-glucans induces greater pulmonary toxicity than soluble 1-->3-beta-glucans in rats. *J. Toxicol. Environ. Health A* 66:25–38.

Zhang, G. H., Baek, L., Buchardt, O., and Koch, C. 1994. Differential blocking of coagulation-activating pathways of limulus amebocyte lysate. *J. Clin. Microbiol.* 32:1537–1541.