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CELL WALL PREPARATIONS FROM ENVIRONMENTAL YEASTS: EFFECT ON ALVEOLAR MACROPHAGE FUNCTION *IN VITRO*

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Abstract: Organic dust toxic syndrome (ODTS) is associated with inhalation of high concentrations of organic materials and is a noninfectious illness characterized by fever, malaise, myalgia, and neutrophilic inflammation of the lower respiratory tract. Studies in our laboratory of fungi in fresh lumber have demonstrated that yeasts may predominate and have raised the issue of potential exposure of sawmill workers to yeasts. Zymosan, a cell wall preparation from *Saccharomyces cerevisiae*, is a potent stimulator of alveolar macrophages (AM). In the present study, preparations from the cell walls of *Pichia fabianii*, *Candida sake*, *Trichosporon capitatum*, *Rhodotorula glutinis*, and *Cryptococcus laurentii* were compared with zymosan and β-1,3-glucan for their ability to stimulate AM and activate complement. All species activated complement. *P. fabianii*, *C. sake*, *T. capitatum*, *R. glutinis*, *C. laurentii*, as well as zymosan and glucan, stimulated superoxide anion and leukotriene B_4 production in a dose-dependent fashion, but *R. glutinis* and *C. laurentii* were much less active. Zymosan, glucan, *P. fabianii*, and *R. glutinis* treatment of AM resulted in increased phagocytosis of labeled sheep RBCs, whereas there was no effect with *C. sake* or *C. laurentii* and *T. capitatum* significantly inhibiting phagocytosis. These results suggest that exposure to high concentrations of yeast could provoke pulmonary inflammation resulting in an episode of ODTS.

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INTRODUCTION

Organic dust toxic syndrome (ODTS) and hypersensitivity pneumonitis (HP) are associated with inhalation of high concentrations of organic materials, particularly agricultural materials such as grain dust, hay, or silage contaminated with microorganisms [9, 13, 18, 19]. Agricultural dusts associated with ODTS characteristically contain large numbers of fungi and bacteria [2, 10, 15]. ODTS is a noninfectious illness resembling the "flu" and is characterized by fever, malaise, myalgia, and a neutrophilic inflammation of the lower respiratory tract [9, 16]. HP (e.g., farmers' lung disease) has many features in common with ODTS including similar exposure settings and clinical symptoms. Although both illnesses appear to involve inflammation of lung parenchyma, they may not be mediated by the same mechanisms. Notably, HP is characterized by a lymphocytic infiltrate into the lower airways suggesting that it may be due to a cell mediated hypersensitivity reaction. Outbreaks of ODTS are characterized by a much higher "attack rate" than is observed in farmer's lung disease; there is usually no correlation between presence of precipitating antibodies and illness; and clinical findings suggest that ODTS

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results from nonspecific immune mechanisms. Studies among farmers in Sweden and in Finland have suggested that ODTS occurs 30–50 times more commonly than farmer's lung disease [6, 12].

Early microbial colonization of stored timber is accomplished by both bacteria [4, 7, 11, 20] and fungi [4, 7, 11], including many yeasts and yeast-like fungi. In an earlier study from our laboratory, we demonstrated that the fungal biota of six species of apparently undecayed timber logs stored for processing in a sawmill consisted largely of yeasts [29].

Microorganisms could be important potential causes of respiratory disorders in woodworkers if inhaled with the sawdust during debarking and sawing operations - e.g., *Cryptostroma corticale* produces a disease known as maple stripper's disease [32]. Studies in our laboratory with spores of common fungi demonstrate that the spores stimulate alveolar macrophages to produce chemotactic substances and inflammatory agents [24, 25, 30, 31]. Glucans represent major structural components of fungal cell walls [21] and recent reports indicate the potential role of fungal glucans as inducers of a chronic pulmonary disease [22]. The objective of the present study is to compare the inflammatory potential of cell wall preparations made from common environmental yeasts with commercial zymosan and glucan.

MATERIALS AND METHODS

Source of yeasts. *Pichia (Hansenula) fabianii*, *Rhodotorula glutinis*, *Cryptococcus laurentii*, and *Candida sake* were isolated from timber logs as part of a study of the microbiota of logs [29]. *Trichosporon capitatum* (NRRL Y-1487) and *Candida sake* (NRRL Y-1622) were obtained from the Midwest Area National Center for Agricultural Utilization Research in Peoria, Illinois.

Cell wall preparations. The procedure used to make the cell wall preparations studied was that described by Pillemer *et al*. [17]. Briefly, the cells were suspended and boiled in 0.5 M Na_2HPO_4 for 3 hr, cooled to 37°C and subjected to repeated trypsin treatments under toluene for 16 days. The pH was adjusted daily to 7.8–8.0. The sediment was collected after centrifugation and washed repeatedly with water, resuspended and washed in absolute ethanol, dried *in vacuo*, refluxed in absolute ethanol, and dried *in vacuo*. The final product was stored under desiccant at 4°C. Each preparation was demonstrated to be endotoxin-free by the Limulus amebocyte lysate analysis (Kinetic QCL; Biowhittaker, Inc., Walkerville, MD). For each experiment, products were suspended in PBS without Ca^{++} or glucose, heated in a boiling water bath for 1 hr, and centrifuged for 30 min at 4000 rpm. After discarding the supernatant fluid, the sediment was resuspended to the concentrations needed in the appropriate diluent for the intended experiment. For comparison, zymosan and β-glucan were purchased from Sigma (St. Louis, MO). Both of these agents are prepared commercially from *Saccharomyces cerevisiae.*

Alveolar macrophage isolation and culture. Alveolar macrophages (AM) were harvested from Sprague-Dawley rats by tracheal lavage. Animals were anesthetized by intra peritoneal injection with sodium pentobarbital and exsanguinated by cutting the renal artery. The lungs of each animal were lavaged with a total of 50 ml of prewarmed calcium- and magnesium-free Hanks balanced salt solution (HBSS). The cells from several animals were pooled, centrifuged at 500 g for 10 min, and washed with PBS. Supplements added to the medium included 10% heat-inactivated fetal bovine serum (HI-FBS); penicillin (P), 100 units/ml; and streptomycin (S) 100 μg/ml. Cell concentrations were determined by hemocytometer. AM suspensions were cultured at a cell concentration of 1×10^6/well in 24 well tissue culture plates (Linbro, Hamden, CO). AM monolayers were incubated for at least 45 min at 37°C in 5% CO_2 to allow for adherence of AM, rinsed with PBS to remove nonadherent cells, and incubated in the presence of cell wall preparations in growth medium. Choice of growth medium and conditions was dependent on the desired assay as described below. Unless indicated otherwise, the cultures were incubated at 37°C in 5% CO_2 for all experiments.

Production of human complement C5a des Arg. The ability of different zymosans to activate complement was studied by monitoring the production of C5a des Arg from normal human plasma by radioimmunoassay. To monitor *in vitro* complement activation, 300 μl portions of zymosan suspensions in saline were placed in tubes with 500 μl normal human plasma and incubated for 30 min at 37°C. After centrifugation, the supernatant fluids were stored at -85°C. Radioimmunoassay was performed according to the procedures outlined by the manufacturer (Amersham International, Amersham, UK) and the results were expressed as ng C5a des Arg per tube.

Superoxide anion production. Rat AMs were obtained as described above, and washed and resuspended in HEPES buffered medium (pH 7.4) at a final concentration of 1.0×10^6 cells/cuvette. Superoxide was measured spectrophotometrically based on reduction of cytochrome C at a wavelength of 550 nm using a Gilford Response II UV-VIS spectrophotometer and Brinkmann Lauda K-2/R circulating water bath at 37°C. HEPES and cells were added to sample and reference cuvettes and after allowing 10 min for equilibration, cytochrome C was added to both cuvettes and superoxide dismutase (SOD) was added to the reference cuvettes only. The final concentrations of cytochrome C and SOD were 120 μM and 2000 units/ml, respectively. Thus the reference and sample cuvettes were identical except that the reference cuvettes contained SOD. Cytochrome C (from horse heart) and SOD (from bovine erythrocytes) were obtained

from Sigma Chemical Co. (St Louis, MO). The kinetic analysis program (version 1.3) was used to make measurements in 6 cuvettes (reference and sample cuvettes for each of 3 samples) 3 times per min. The program automatically corrects for absorbance in the reference cuvette (untreated control). Resting rates were determined for each pair of cuvettes during the first 4 min. Varying quantities of zymosans were added to both cuvettes and changes in absorbance were recorded for the next 15 min. Amounts of superoxide anion produced were calculated by determining the change in absorbance between the time of addition of sample and 15 min after addition of sample, and results were expressed as nmol $O^-_2/1.0 \times 10^6$ cells/15 min.

Production of leucotriene B_4 (LTB_4). After removal of nonadherent cells from the AM monolayers, the medium was replaced with RPMI (Roswell Park Memorial Institute) 1640 + 0.1% bovine serum albumin (BSA), P, S, and glutamine ± freshly prepared zymosans and incubated under standard conditions. AM-conditioned media were collected after 3 hr incubation of AMs in RPMI 1640 with zymosans. Samples of the media were filtered through a 0.45 μm filter and stored at -80°C prior to analysis. Enzyme-linked immunosorbent assay (ELISA) was performed according to the procedures outlined by the manufacturer (PerSeptive BioResearch, Cambridge, MA) and results were expressed as ng of LTB_4 per ml of medium.

Production / release of tumor necrosis factor (TNFα). TNFα was measured by the method of Shahan *et al.* [23] except that MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was used as an indicator of cell viability. The cells used in the assay were derived from a clone of the murine fibroblastoid cell line L929 incubated in Iscove's modified Dulbecco's medium containing sodium bicarbonate (7.5% w/v), heat inactivated bovine serum (10%), and 0.5 μg/ml actinomycin D. Cells were incubated in the presence of a range of concentrations of Murine rTNFα or sample. After 20 hr incubation, 30 μl of MTT (2.5 mg/ml in PBS) was added and the plates were incubated an additional 4 hr. Then the plates were centrifuged at 500 g in a 96-well plate holder (Eppendorf model 5403 centrifuge, Brinkmann Instrument, Inc. Westbury, NY), the medium removed by aspiration and 100 μl dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan crystals. Optical density was read at 570 nm using a Dynatech ImmunoAssaySystem plate reader (Dynatech, Chantilly, VA). Determination of TNF in samples was accomplished by best fit regression (sigmoid) of standard curves on the same plate (TMS software version 1.63).

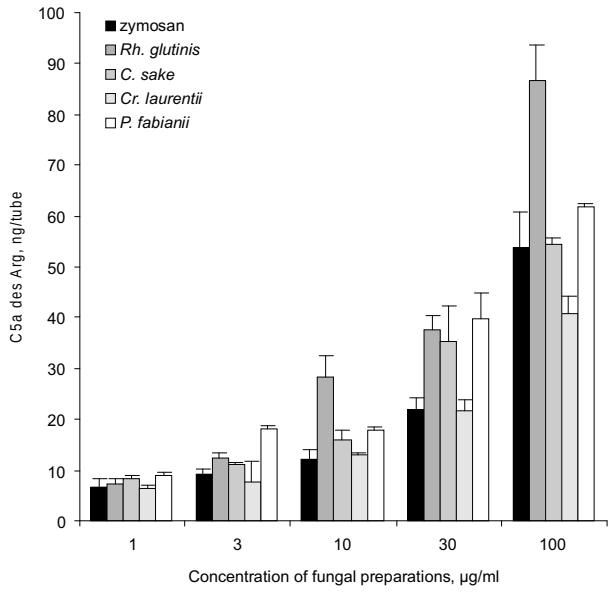

Figure 1. Activation of human complement by different species of environmental yeast. The value for the untreated control was 5.8 ± 0.29 ng/tube (mean ± SEM).

Phagocytosis of ^{51}Cr-labeled sheep erythrocytes. AM monolayers were prepared in Linbro tissue culture plates, nonadherent cells were removed, and the plates were incubated overnight. Phagocytosis was assessed according to the procedure of Snydermann *et al.* [27]. After a 3 hr incubation with and without zymosans, the medium was removed and 0.5 ml of fresh medium containing 2×10^7 ^{51}Cr-labeled sheep red blood cells was added. The monolayers were then incubated for 1 hr. After incubation, the medium was removed and the bound extracellular sheep cells were lysed with lysing medium. The monolayers were washed twice more with lysing medium, dissolved with 0.5% sodium dodecyl sulfate (SDS), and counted by gamma scintillation.

Statistical analysis. One-way analysis of variance was used to compare dose levels in dose-response curves and the Student's t-test was used to compare the means of the experimental groups to the respective controls. The 5% significance level was used in both instances.

RESULTS

Activation of complement. Yeast cell wall preparations from all sources tested were able to activate complement in a dose-dependent manner as evidenced by production of C5a des arg (Fig. 1). Differences between dose levels were highly significant ($p < 0.001$) by one-way ANOVA for each cell wall preparation tested including glucan and *T. capitatum* which are not shown in Figure 1.

Superoxide anion production. Superoxide anion production varied greatly according to species (Fig. 2). The most striking response was observed with material from *T. capitatum* which reached a peak at 7.5 mg/ml, whereas the peak response of most other samples occurred at 25 mg/ml. Zymosan and glucan produced

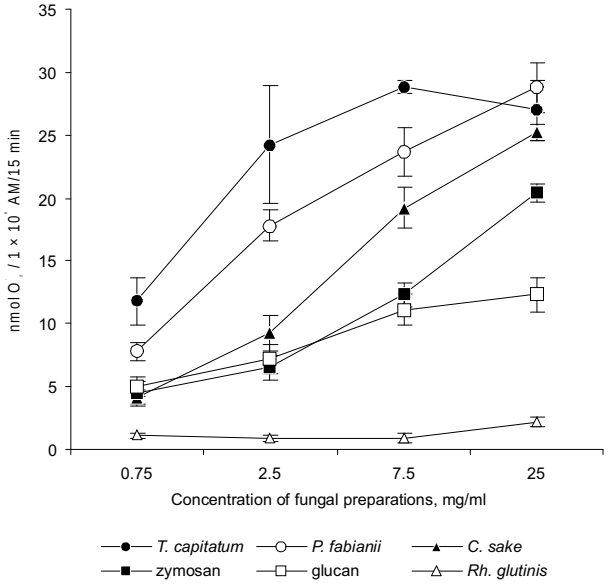

Figure 2. Stimulation of macrophage superoxide anion production with environmental yeasts (mean ± SEM).

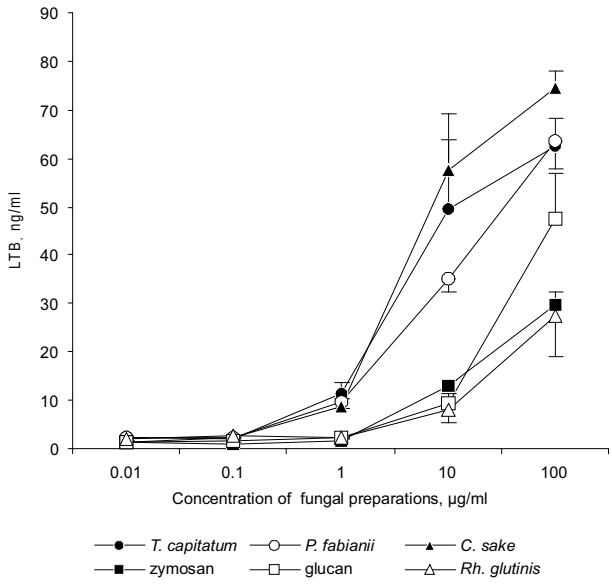

Figure 3. Stimulation of macrophage LTB_4 production by different species of environmental yeast (mean ± SEM).

responses similar to that of *C. sake* but somewhat lower than *P. fabianii*. There was little response to the products from *Rh. glutinis* or *Cr. laurentii* (data not shown). The program used for this analysis reads sample and untreated control cuvettes simultaneously and automatically corrects for control. Therefore, no data are generated for controls, but one-way ANOVA demonstrated that differences between dose levels were highly significant ($p < 0.001$) for all species except *T. capitatum* ($p = 0.048$), *Rh. glutinis* ($p = 0.072$) and *Cr. laurentii* ($p = 0.249$). The physical properties of the material from *T. capitatum* made reproducibility difficult.

LTB_4 production. All of the cell wall preparations studied were able to stimulate production of LTB_4 by AM, but response varied with the organism and was lowest with the basidiomycete yeasts studied, i.e., *Rh. glutinis* and *Cr. laurentii* (data not shown). LTB_4 was detected in cultures treated with *Cr. laurentii* and *Rh. glutinis* at the 100 µg/ml level only. The greatest LTB_4 response was observed in the cultures treated with preparations from *C. sake*, *T. capitatum* and *P. fabianii*, with lesser amounts produced after stimulation with zymosan or glucan. Maximum production with these preparations were observed when the cells were treated with 100 µg/ml and there was a dose-dependent response between 1 and 100 µg/ml (Fig. 3). Differences between dose levels for all species were highly significant by one-way ANOVA ($p < 0.001$).

TNF. In general, cell wall preparations from all species of yeast tested stimulated production and release of TNF-α but the response varied greatly with the species (Fig. 4). The positive control (LPS, 1 µg/ml) stimulated production of ca. 15.4 ng/ml of culture medium and was not tested at higher levels. When the macrophages were treated at a concentration of 100 µg/ml of cell wall preparation, 79.1, 69.2, 3.5 and 1.3 ng/ml were found in the culture medium for zymosan, *P. fabianii*, *T. capitatum* and glucan respectively. Thus, *P. fabianii* was essentially equal to zymosan in its effect. The preparation from *Rh. glutinis*, at a dose of 100 µg/ml, gave a response approximately equal to that of the positive control but the response at lower doses was comparable to the negative control (ca. 10 pg/ml). Most samples produced dose-dependent increases in TNF but, with the exception of *Rh.*

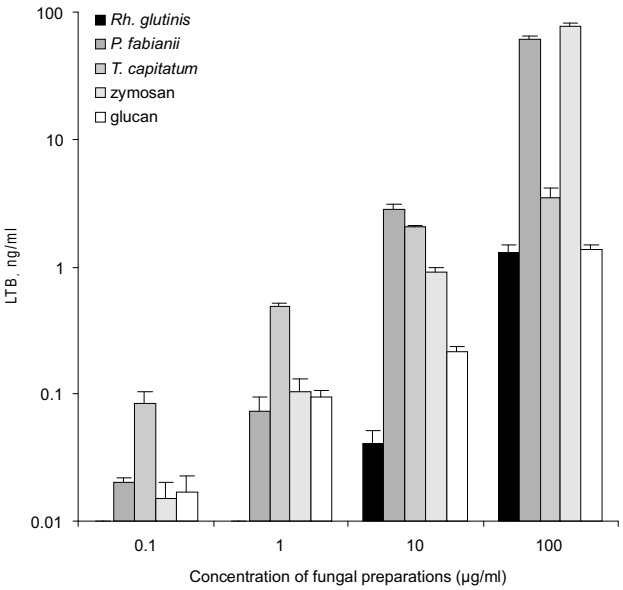

Figure 4. Production and release of tumor necrosis factor-α (TNF-α) following stimulation with cell wall preparations of environmental yeasts (mean ± SEM). The response for the positive control (LPS, 1.0 µg/ml) was 15.4 ± 0.2 ng/ml (data not shown).

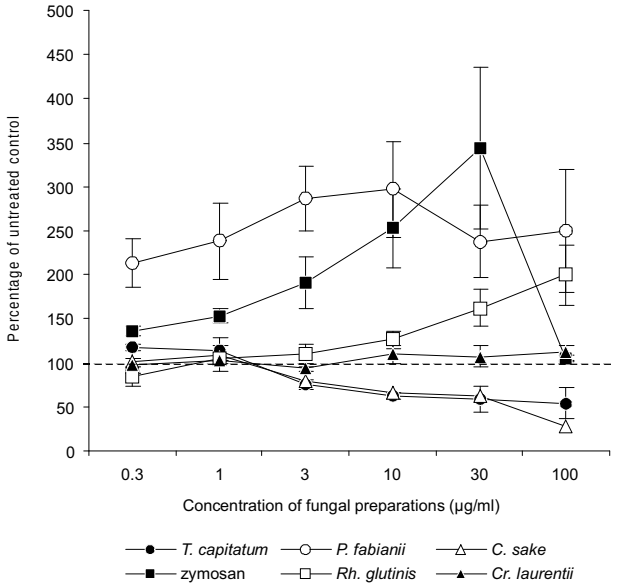

Figure 5. Effect of yeast cell wall particles on phagocytosis of labeled sheep erythrocytes. The dotted line indicates the response of untreated control cells and values for fungal preparations are given as percentage of control. The response curve for glucan (data not shown) was nearly identical to that of zymosan but response was slightly lower at all doses.

glutinis, maximum yields were well below 1.0 ng/ml (data not shown). For example, whereas ca. 0.5 ng/ml was stimulated at a dose of 100 µg/ml of *Candida sake*, the response to all other doses of this sample and all doses of *Cr. laurentii* were less than 0.1 ng/ml. TNF concentrations in the agonist-controls, i.e., samples prepared with 100 µg/ml of each of the cell wall preparations incubated in culture medium without the presence of alveolar macrophages, were comparable to the negative control. Analysis of differences between dose levels by one-way ANOVA was highly significant ($p < 0.001$) for all species except *Cr. laurentii* ($p < 0.25$).

Phagocytosis. Phagocytosis was stimulated in a dose-dependent manner by both of the commercial preparations, i.e., zymosan and glucan, up to a peak stimulation at a concentration of 30 µg/ml where the response was >300% of that observed for untreated control cells (Fig. 5). At a concentration of 100 µg/ml, response was ca. 100% to 150% of control for zymosan and glucan, respectively. The *P. fabianii* preparation produced the greatest stimulation at low levels; it peaked at ca. 300% of control at 10 µg/ml and remained above 200% of control at 30 and 100 µg/ml respectively. The preparation from *Rh. glutinis* produced a response of ca. 150% and 200% of control at concentrations of 30 and 100 µg/ml respectively, whereas that from *Cr. laurentii* produced a response similar to that of the untreated controls. The preparations from *T. capitatum* and *C. sake* (two strains), on the other hand, inhibited phagocytosis at the higher concentrations tested (Fig. 5). The stimulation observed with high doses of zymosan, glucan, *P. fabianii*, and *Rh. glutinis* was highly significant by the Student's t-test when raw data from untreated controls and samples were compared ($p < 0.001$). Similarly, the inhibitory effect of high doses of extract from *C. sake* (both strains) and *T. capitatum* was highly significant ($p < 0.001$).

DISCUSSION

Recently, several investigators have reported macrophage activation or inflammation in response to yeast, including enhanced phagocytosis and oxidative metabolism in alveolar macrophages treated with *Candida albicans* and *Saccharomyces cerevisiae* [14], activation of macrophages and specific T lymphocytes in mice inoculated intratracheally with *C. neoformans* [8] and production of TNF-α in human leukocytes stimulated with *C. neoformans* [10]. Others have provided evidence that β-1,3-glucans are involved in this activation. For example, Castro *et al.* [1] hypothesized that macrophages respond to live or dead *C. albicans* by releasing arachidonic acid (AA) and AA metabolites as a consequence of interaction of mannose and β-1,3-glucan receptors with fungal cell wall components. Treatment of the macrophages *in vitro* in the presence of *C. albicans* with soluble α-mannose or, to a lesser degree, with soluble β-glucan, significantly inhibited this stimulation of AA metabolism indicating that *C. albicans* stimulation of AA metabolism is partly mediated by α-mannose and β-glucan constituents of the fungus. Similarly, Silva and associates [26] demonstrated that cell wall preparations containing predominantly β-glucan injected into the peritoneal cavity of rats induced an inflammatory response similar to that observed during the initial stage of paracoccidioidomycosis as well as in experimentally infected animals, suggesting that β-glucans may play a role in the virulence and pathogenicity of *Paracoccidioides brasiliensis.* It is believed that macrophage activation is the central event in the β-glucan immune stimulatory effect [34].

ODTS is associated with exposure to very high levels of microbial contamination in organic materials, e.g., moldy straw or hay, and Rylander [22] has provided evidence for the involvement of glucan in airway disease in a day-care center in Sweden. Investigations in our laboratory of spores from fungi isolated from such materials have revealed similar activation of macrophages including stimulation of superoxide anion, LTB_4, TNF-α, activation of complement, and production of chemotactic factors [25, 30, 31]. Shahan *et al.* [24] demonstrated upregulation of macrophage inflammatory protein (MIP-2) and KC, members of the C-X-C family of cytokines, when alveolar macrophages were exposed to conidia of *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus candidus* and *Eurotium amstelodami.* It has been suggested that KC and MIP-2 are the rat physiological equivalents of interleukin (IL)-8 and GRO (9). Of the fungi studied, only *A. fumigatus* upregulated mRNA transcripts for TNF-α.

In the present study, it was demonstrated that cell wall preparations from a variety of environmental yeasts isolated from logs in a lumber yard activate complement, stimulate production of superoxide anion, LTB_4, and TNF-α, and also influence phagocytic response. These findings are in agreement with those of other investigators who have studied activation of macrophages by yeast and are consistent with the hypothesis that yeasts could contribute to the development of ODTS. Shahan *et al.* [24], in a study of stimulation of macrophages by intact fungal spores of several different species, showed that only spores of *A. fumigatus* stimulated increases in mRNA's for TNF-α. In contrast, all the yeast species in the present investigation stimulated TNF-α production. Of interest is the observation that the yeast cell wall extracts tended to be more potent activators than the glucan preparation. This finding suggests that other cell wall components may contribute to the activation process. The demonstration that yeasts may constitute the predominant fungi in fresh lumber and the fact that they have inflammatory potential suggests that they could contribute to the development of occupational lung disease among sawmill workers.

CONCLUSION

Cell wall preparations from a variety of yeasts isolated from fresh lumber were shown to have inflammatory activities in rat alveolar macrophage culture. All species studied activated complement and stimulated leucotriene B_4 production. Both these activities have important consequences for chemotaxis. *Trichosporon capitatum*, *Pichia fabianii* and *Candida sake*, as well as commercial zymosan and glucan, stimulated superoxide anion and tumor necrosis factor-α production but the magnitude of the response varied with the species. Phagocytosis was stimulated by zymosan, glucan, *P. fabianii*, and, to some extent by *Rh. glutinis*, but was unaffected by *Cr. laurentii* and was inhibited by *T. capitatum* and *C. sake*. The basidiomycete yeasts, *Rhodotorula glutinis* and *Cryptococcus laurentii*, were generally the least active.

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