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Superoxide Anion Production in Response to Bacterial Lipopolysaccharide and Fungal Spores Implicated in Organic Dust Toxic Syndrome

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High amounts of fungal spores, bacteria, and bacterial endotoxin have been found in dust associated with the poorly characterized syndrome, organic dust toxic syndrome (ODTS). As part of an ongoing investigation to determine the etiopathogenesis for ODTS, this study has focused on activation of guinea pig bronchial alveolar lavage (BAL) cells as evidenced by the production of superoxide anion in response to fungal spores and lipopolysaccharide (LPS). Fungal spores from *Aspergillus candidus, Aspergillus terreus, Aspergillus niger, Aspergillus fumigatus, Eurotima amstelodami, Penicillium spinulosum,* and *Cladosporium cladosporioides* were all shown to increase superoxide anion production, each with different potencies. LPS stimulated little superoxide anion production in BAL cells, but when cells were pretreated with LPS prior to stimulation with fungal spores, superoxide anion production was increased over that induced by either spores or LPS alone. These results suggest that the inhalation of LPS together with fungal spores could possibly provoke abnormal lung pathologies.

INTRODUCTION

Organic dust toxic syndrome (ODTS) is associated with the inhalation of high concentrations of organic materials, such as grain dust, hay, silage, or haylage contaminated with microorganisms. ODTS is characterized by development of acute respiratory, flu-like symptoms, including headache, myalgia, and malaise. ODTS is distinguished from hypersensitivity pneumonitis by the identification of neutrophilic infiltration in the lung, case clustering, and rapid onset as well as negative serology (May *et al.*, 1990; Malmberg, 1990). Although the exact cause of ODTS is unknown, it is thought that the sequelae seen in ODTS are due to nonantigen-specific activation of the immune system by microorganisms found in organic dusts, i.e., gram-negative bacteria and fungi.

Experimental inhalation of endotoxin or lipopolysaccharide (LPS) has been shown to cause the following: (i) an increase in pulmonary prostaglandin synthesis, (ii) hyperoxia and pulmonary edema, (iii) increased lung resistance and pulmonary arteriole pressure with decreased lung compliance, (iv) sequestration of peripheral blood leukocytes into pulmonary capillaries, (v) infiltration of leukocytes into pulmonary interstitium with focal endothelial damage, and (vi) neutrophil infiltration into the alveoli (Burrell *et al.*, 1988; Rylander, 1987).

The mechanisms of priming of macrophages by LPS is not completely understood, but it affects many of the functional capacities of cells (Sasada *et al.*, 1993).

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Many of the physiological effects of endotoxin or its purified derivative, LPS, may be mediated by macrophages (Adams *et al.*, 1992) which generate potent mediators of shock such as IL-1, platelet activating factor, tumor necrosis factor, and IFN-γ (Jue *et al.*, 1990; Terashita *et al.*, 1985; Heremans *et al.*, 1990) in response to LPS.

Macrophages and endothelial cells which line the airways are the primary lines of defense. Stimulation of macrophages results in an increase in oxygen consumption and cellular respiration (Block, 1992). The products of increased cellular respiration are reactive oxygen metabolites, degradative enzymes, cytokines, and H^+ ions. These substances, although essential for the elimination of foreign substances, can also be detrimental to host tissues. Lysosomal enzymes and reactive oxygen metabolites may cause damage or death to neighboring cells leading to the release of other immunological mediators which could activate other immunological effector mechanisms (Block, 1992; Brain, 1992; Ward, and Johnson, 1986).

Aspergillus niger and *A. fumigatus* have been shown to produce substance(s) that caused a dose-dependent inhibition of superoxide anion production in bronchial alveolar lavage (BAL) cells (Robertson *et al.*, 1987; Sorenson *et al.*, 1992). Killing the spores by autoclaving or formalin inhibits the ability of the cell to make or to destroy these inhibitory substance(s) if it is already present in the cell. Fungal spores have also been shown to activate complement by the alternative pathway causing the production of complement component C5a, a potent anaphylatoxin (Sorenson *et al.*, 1992). Spores have also been shown to initiate the production of the arachidonic acid metabolite leukotriene B_4 (Sorenson *et al.*, 1992). Although evidence is lacking, it is possible that fungi and endotoxin as well as the grain and hay dust component of organic dust may work in synergy with each other to cause disease. This paper will focus on the effects of fungal spores and LPS on superoxide anion production and BAL cell activation in guinea pig BAL cells.

MATERIALS AND METHODS

Reagents Used

Cytochrome c from bovine erythrocytes, superoxide dismutase (SOD), phorbol myristic acetate (PMA), and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). PMA was dissolved in DMSO. Hank's balanced salt solution (HBSS) with and without Ca^{2+} and Mg^{2+} was obtained from Life Technologies Inc. (Gaithersburg, MD). LPS omega 0128:B12 was obtained from Difco (Detroit, MI).

Fungal Spores

Aspergillus fumigatus Fresen., *A. niger* Tiegh., *A. terreus* Thom., *A. candidus, Eurotima amstelodami,* and *Penicillum spinulosum* Thom. were isolated from material associated with outbreaks of ODTS and maintained by lyophilization. Fresh cultures were revived from lyophilized preparations for use in experiments and inoculum was prepared from cultures grown 7 days on malt extract agar. Spores were removed from culture tubes by adding 4.0 ml HBSS containing 0.01%

Tween 80, vortexed, and scraped with a pipette to loosen spores. Filtration of these suspensions through several layers of sterile gauze resulted in suspensions containing predominantly unclumped spores with no hyphae or conidiophores. Conidial suspensions were washed five times in HBSS by centrifugation (3200*g* at 4°C) to remove the residual Tween 80 and conidial diffusates. Macrophage and spore concentrations were determined using a Neubauer hematocytometer and results confirmed using a Coulter counter with channelizer (Coulter Corporation, Model ZM, Hialeah, FL).

Spores were killed by autoclaving in phosphate-buffered saline (PBS) at 121°C, 15 PSI for 30 min and washed five times with PBS.

BAL Cell Isolation

BAL cells were harvested from English Short Hair Guinea Pigs (strain LAF-1, Camm Research Animals, Camden, NJ) by bronchoalveolar lavage. Animals were anesthetized by intraperitoneal injection with sodium pentobarbital and exsanguinated by cutting the renal artery. The lungs of each animal were lavaged with a total of 80.0 ml of prewarmed HBSS w/o Ca^{2+} and Mg^{2+}. The cells from several animals were pooled, centrifuged (1250*g* for 15 min at 4°C), and washed with HBSS with Ca^{2+} and Mg^{2+}. Guinea pig BAL cells were washed and resuspended in HBSS containing Ca^{2+} and Mg^{2+} to achieve a final concentration of 2.5×10^5 cells/well.

Superoxide Anion Production: Micro Procedure

Superoxide anion was measured spectrophotometrically based on the reduction of cytochrome c at a wavelength of 550 nm using a 96-well plate reader. Reaction mixtures contained 120 μM cytochrome c, ±1.0 μg/ml PMA, ±833.0 units/ml SOD, and quantities of spores or other agents that varied depending on the question being addressed. The 96-well reaction plate (Costar, 3596, Cambridge, MA) was incubated (37°C, CO_2 incubator, 5% CO_2) on a 96-well plate shaker (TiterTek, Germany) which was used to keep the BAL cells and stimulants in suspension. Measurements were made from each well at 0 min (immediately following mixing after the addition of cells, stimulants, and cytochrome c) and 30 min to control for variations in optical density due to varying concentrations of stimulants and the different opacities of the stimulus. For pretreatment experiments 100 ng *Escherichia coli* LPS omega (0128:B12), fungal spores, or PMA (0.2 μg/ml) were incubated with BAL cells for 30 min (37°C, 5% CO_2). At 0 min 100 μl of the reaction mixture was transferred to the same well location in a new 96-well plate. At 30 min the rest of the reaction mixture was removed from the reaction plate and again placed into a new 96-well plate. The plates were centrifuged (1500*g* for 15 min), supernatants were removed, and 75 μl was added to a new 96-well plate. Control wells contained no spores or LPS. Superoxide anion production was calculated by measuring the change in absorbance over time in experimental wells minus the change in absorbance over time in corresponding wells containing SOD. The results expressed as nmol are actually nmol $O^-{}_2/2.5 \times 10^5$ cells/30 min but abbreviated to conserve space.

Statistical Analysis

All data were expressed as mean ± SEM. Comparisons between the dead and live spores were made using a modified Student's t test. Data were considered statistically significant if P values were <0.05 for two-sided alternatives. All other statistical analyses with multiple comparisons were performed using Tukey's honestly significant difference test (Dowdy and Wearden, 1983). Data were considered statistically significant if q values were <0.05.

RESULTS

In Vitro Effect of Fungal Spores on Superoxide Production

Spores from each of the fungi studied were able to cause increases in superoxide anion production without additional stimulus (Figs. 1–4). Production of superoxide anion varied with each species and ranged from 1.4 ± 0.19 (*E. amstelodami*) to 4.6 ± 0.73 nmol (*A. candidus*). There was no significant difference between live and dead spores in their ability to stimulate superoxide anion production (Fig. 2, all P values >0.137). Washed spores from all fungi showed a density-dependent increase in superoxide anion production similar to that illustrated in Fig. 1 for *A. niger* and *A. fumigatus*, although total amounts of superoxide anion produced

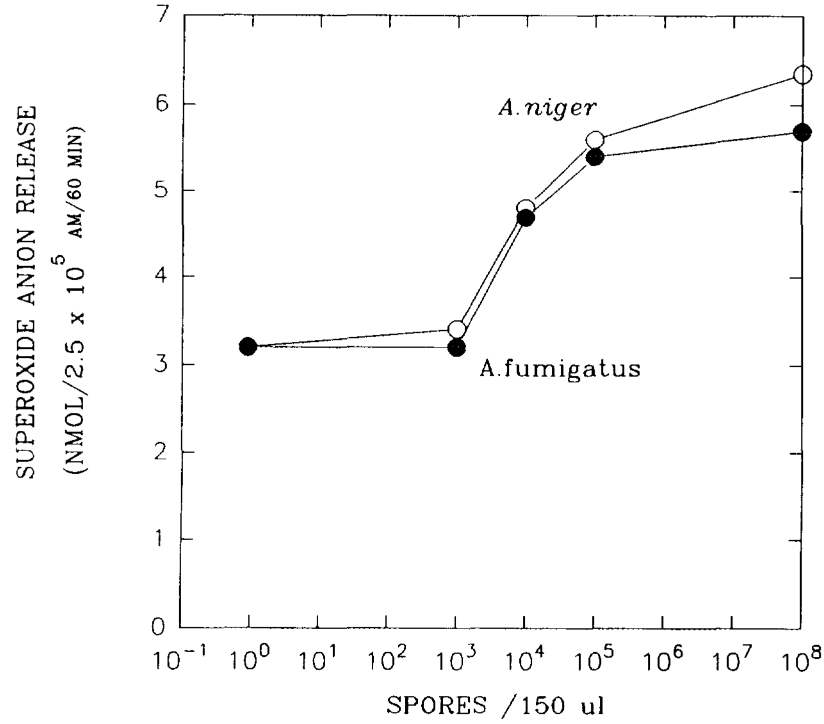

FIG. 1. Dose–response characteristics of BAL cells to *A. niger* and *A. fumigatus* fungal spores. Superoxide anion release was assayed by analyzing the reduction state of cytochrome c with a 96-well plate reader equipped with a 570-nm filter. Cell only control = 3.2 ± 0.47 nmol.

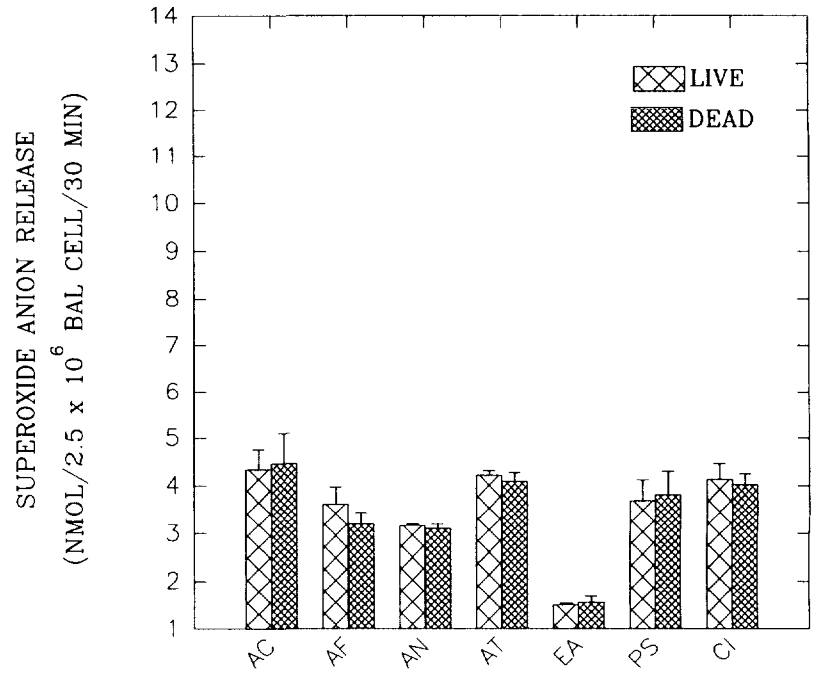

FIG. 2. Response characteristics of BAL cells to live and dead (autoclaved) fungal spores. Superoxide anion release was assayed by analyzing the reduction state of cytochrome c with a 96-well plate reader equipped with a 570-nm filter. Data represent means ± SEM (N = 10). AC, *A. candidus;* AF, *A. fumigatus;* AN, *A. niger;* AT, *A. terreus;* EA, *E. amstelodami;* PS, *P. spinulosum;* Cl, *C. cladosporioides.* Cell only control = 3.2 ± 0.47 nmol.

varied with the species. Treatment of BAL cells with LPS and *A. niger* spores together stimulated 5.6 ± 0.68 nmol of superoxide anion.

Prestimulation (Priming) of Guinea Pig BAL Cells

Spores from each of the fungal species studied caused greater increases in superoxide anion production in LPS-primed BAL cells than in those with no priming. Production of superoxide anion by LPS-primed BAL cells varied with the fungal species and differences ranged from 3.8 ± 0.43 nmol (*E. amstelodami*) to 10.8 ± 1.07 nmol (*A. niger*). In experiments without LPS priming, the values ranged from 1.4 ± 0.19 (*E. amstelodami*) to 4.6 ± 0.73 nmol (*A. candidus*). Comparison of fungal spore stimulation of both LPS-primed and nonprimed BAL cells demonstrated a significant increase ($P < 0.05$, using the t test) in superoxide anion production in pretreated cells compared to that in nonpretreated cells, except for *P. spinulosum* and *A. candidus* (Fig. 3). Pretreatment with *A. niger* followed by treatment with LPS again caused a significant increase ($q < 0.008$) in superoxide anion when compared to either tested alone as a pretreatment or treatment (Fig. 4).

The optimum LPS-priming concentration in these experiments was determined by pretreating BAL cells with different concentrations of LPS for 30 min prior to

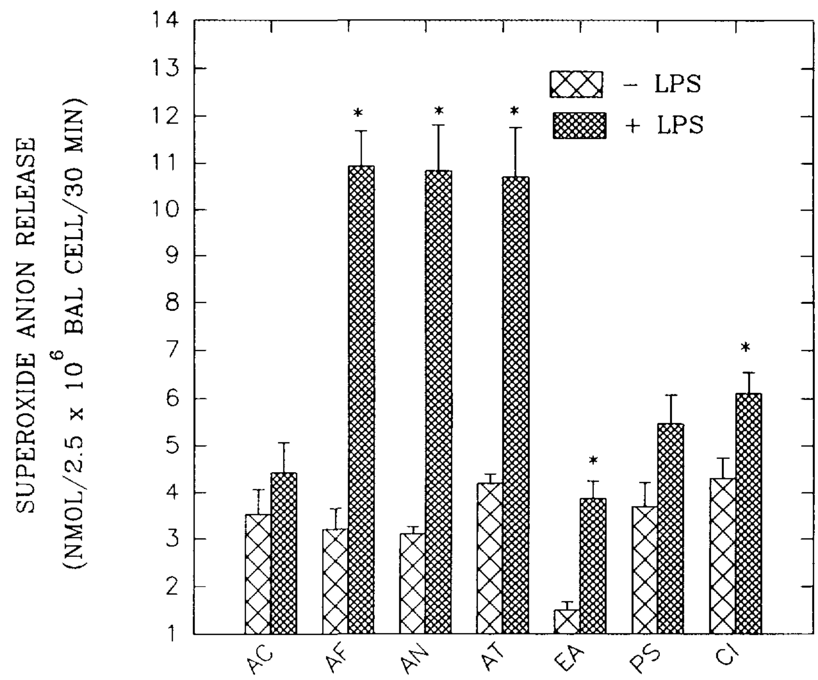

FIG. 3. Response of BAL cells with or without LPS (100 ng) pretreatment for 30 min followed by treatment with fungal spores (1 × 10^8 spores). Superoxide anion release was assayed by analyzing the reduction state of cytochrome c with a 96-well plate reader equipped with a 570-nm filter. Data represent means ± SEM ($N = 10$). *$P < 0.05$ compared with cells without LPS pretreatment. AC, *A. candidus;* AF, *A. fumigatus;* AN, *A. niger;* AT, *A. terreus;* EA, *E. amstelodami;* PS, *P. spinulosum;* Cl, *C. cladosporioides.* Cell only control = 3.2 ± 0.47 nmol.

exposure to 1 × 10^8 spores/150 μl and measuring the reduction state of cytochrome c. This concentration was determined to be 100 ng/ml, which was approximately the lowest concentration of LPS at the plateau of the dose–response curve (Fig. 5). To determine the optimal time for pretreatment or priming, BAL cells were pretreated with 100 ng/ml LPS at five different time intervals (0, 30, 60, 120, and 240 min). After each of these pretreatment intervals the BAL cells were incubated with 1 × 10^8 spores/150 μl for 30 min before measuring the reduction state of cytochrome c. The optimum pretreatment time was determined to be 30 min. Treatment with spores caused no further increase in superoxide anion production after 4 hr of priming with LPS.

Following pretreatment with PMA for 30 min or longer, treatment with any agent stimulated little or no superoxide anion production; however, treatment with PMA only resulted in a large increase in superoxide anion production. When fungal spores from each species were used as a pretreatment and PMA was used as a treatment there was only a 2× increase in superoxide anion release (data not shown).

DISCUSSION AND CONCLUSIONS

The following conclusions were made: (i) fungal spores stimulate cell activation

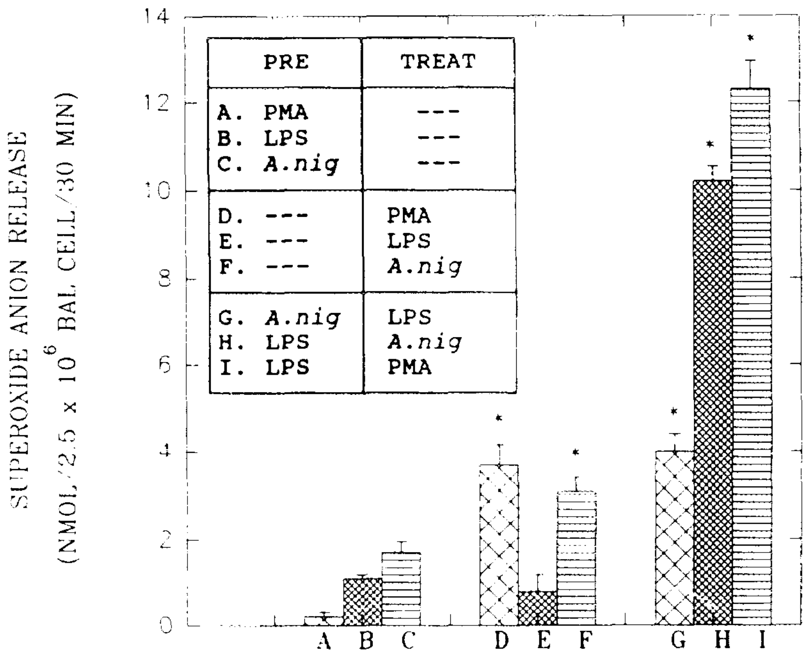

FIG. 4. Response characteristics of BAL cells exposed to pretreatment with PMA, LPS, or *A. niger* (1×10^8 spores) for 30 min alone or followed by treatment with the same agents alone or in sequence. The letters under the bars correspond to the letters in the key. Superoxide anion release was assayed by analyzing the reduction state of cytochrome c with a 96-well plate reader equipped with a 570-nm filter. Data represent means ± SEM. $*q < 0.05$ compared with cells that were exposed to the same agents used either as treatment or pretreatment. *Aspergillus niger* was abbreviated *A. nig* to conserve space. Cell only control = 3.2 ± 0.47 nmol.

and respiratory burst in BAL cells, (ii) cell activation and respiratory burst varies with the fungal species, and (iii) LPS pretreatment primed BAL cells for enhanced release of superoxide anion following treatment with fungal spores.

The only consistently identified components in organic ducts associated with ODTS are fungal spores and bacterial endotoxin. Fungi can be pathogenic as well as allergic. LPS elicits many immunological responses, including the nonspecific activation of complement, B cell activation, kinin system activator, and the potent stimulation of Il-1, IFN-γ, and TNF-α production which are believed to be the major immunological mediators in endotoxic shock and the Schwartzman reaction (Burrell *et al.*, 1988; Burrell, 1990; Brigham and Meyrick, 1986; Heremans *et al.*, 1990).

This investigation demonstrated that bacterial LPS was a poor BAL cell activator by itself, but a very potent primer. Pretreatment with LPS, followed by treatment with fungal spores, caused greater superoxide anion production than that stimulated by spores or LPS individually. These data indicate that the combination of spores and LPS could potentially work in synergy and cause activation of effector cells in the airway. BAL cells treated with a combination of LPS and fungal spores caused increases in superoxide anion production which were not

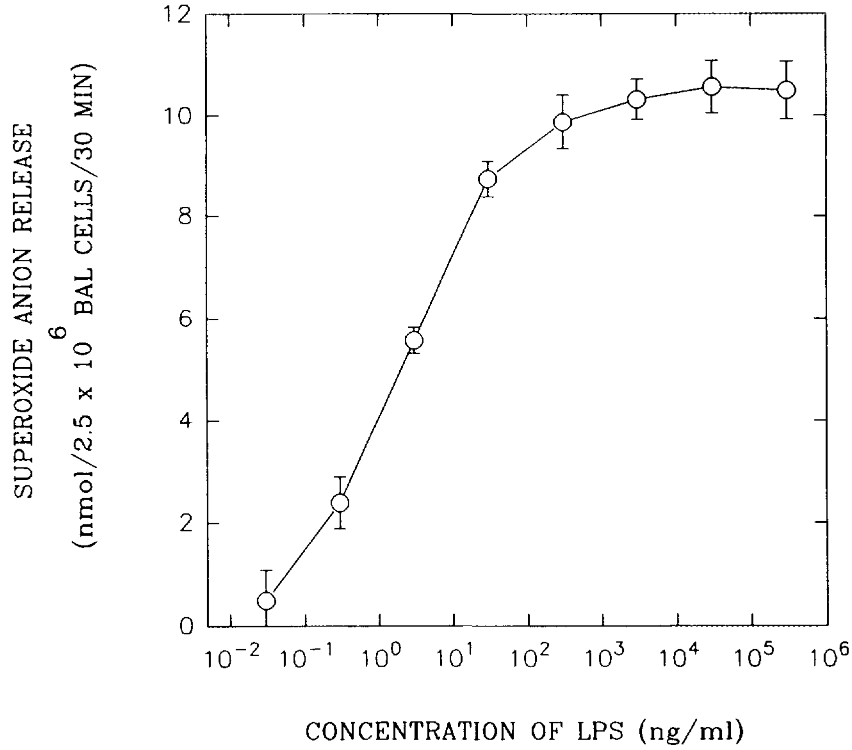

FIG. 5. Dose–response characteristics for superoxide anion production from BAL cells pretreated with varying concentrations of LPS. After pretreating cells with LPS for 30 min the cells were incubated with 1 × 10^8 *A. niger* spores for 30 min. Superoxide anion release was assayed by analyzing the reduction state of cytochrome c with a 96-well plate reader equipped with a 570-nm filter. Data represent means ± SEM. Cell only control = 3.2 ± 0.47 nmol, cells + LPS pretreatment (100 ng/ml) = 1.3 ± 0.079 nmol, cells + *A. niger* pretreatment = 1.85 ± 0.24 nmol, cells + *A. niger* = 2.4 ± 0.41 nmol.

significantly different ($P = 0.127$) than treatment with fungal spores alone (data not shown). In this type of *in vitro* experiment the assumption is made that all cells are in contact with the same concentration of any agent, but this may not be true in the lung. Agents in the environment are often found in different concentrations that can vary with both time and location. Respiratory dynamics as well as immune cell interactions with agents remain largely unexplored making practical interpretation of these data difficult.

Experiments using PMA as a pretreatment followed by treatment with any other agents resulted in no superoxide anion production; however, when PMA was used as a treatment instead of a pretreatment there were large amounts of superoxide anion produced. PMA is a strong cell activator of protein kinase c which is necessary for cell stimulation and effector cell function. We believe that pretreating cells with PMA (1.0 μg/ml) for 35 min caused the cells to become hyperactivated which, in turn, caused them to utilize all of their available resources during pretreatment, so that they were unable to respond when they came into contact with another stimulus.

Suzuki *et al.* (1993) reported that pretreating macrophages with LPS (500 ng/ml) for 24 hr resulted in maximal superoxide anion production when treated with PMA (2.0 μg/ml). At times less than 24 hr, the values for the untreated control animals were usually below the spontaneous values. We found that after 30 min pretreatment with LPS (100 ng/150 ml), there was no further increase in superoxide anion production when cells were treated with fungal spores for a total of 240 min. The length of time we pretreated our cells was 46 hr less than Suzuki pretreated his cells, so it is conceivable that if we had pretreated the cells for 24 hr, superoxide anion production from LPS pretreatment could have been higher.

In conclusion, LPS acts as a priming or enhancing signal for further cell activation by fungal spores. Fungal spores alone have also been shown to be capable of activating BAL cells. Bacteria and fungi are commonly found in organic dusts. If these data are an accurate representation of what actually occurs *in vivo,* this finding may provide a starting point for further work investigating the toxicity of organic dusts.

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REFERENCES

Adams, D. O., and Hamilton, T. A. (1992). Molecular basis of macrophage activation: Diversity and its origins. *In* "The Natural Immune System: The Macrophage" (C. A. Lewis, and J. O. McGee, Eds.). IRL Press, New York.

Block, E. (1992). Pulmonary endothelial cell pathobiology; Implications for acute lung injury. *Am. J. Med. Sci.* **304**, 136–144.

Brain, J. D. (1992). Mechanisms, measurement, and significance of lung macrophage function. *Environ. Health Perspect.* **97**, 5–10.

Brigham, K. L., and Meyrick, B. (1986). Endotoxin and lung injury. *Am. Rev. Respir. Dis.* **133**, 913–927.

Burrell, R., Lantz, R. C., and Hinton, D. E. (1988). Mediators of pulmonary injury induced by inhalation of bacterial endotoxin. *Am. Rev. Respir. Dis.* **137**, 100–105.

Burrell, R. (1990). Immunomodulation by bacterial endotoxin. *Crit. Rev. Micro.* **17**, 189–208.

Dowdy, S., and Wearden, S. (1983). "Statistics for Research" (R. A. Bradley, J. S. Hunter, D. G. Kendall, R. G. Miller, and G. S. Watson, Eds.). Wiley, New York.

Heremans, H., Van Damme, J. O., Dillen, C., Dijkmans, R., and Billiau, A. (1990). Interferon γ, a mediator of lethal lipopolysaccharide-induced Schwartzman-like shock reactions in mice. *J. Exp. Med.* **171**, 1853–1869.

Jue, D. M., Sherry, B., Manogue, K. R., and Cerami, A. (1990). Processing of newly synthesized cachectin/tumor necrosis factor in endotoxin-stimulated macrophage. *Biochemistry* **29**, 8371–8377.

Malmberg, P. (1990). Health effects of organic dust exposure in dairy farmers. *Am. J. Ind. Med.* **17**, 111–113.

May, J., Marvel, L. H., Pratt, D. S., and Coppolo, D. (1990). Organic dust toxic syndrome: A follow-up study. *Am. J. Ind. Med.* **17**, 111–113.

Raz, A., Wychw, A., Siegel, N., and Needleman, P. (1988). Regulation of fibroblast cyclooxygenase synthesis by IL-1. *J. Biol. Chem.* **263**, 3022–3028.

Robertson, M. D., Seaton, J. A., Raeburn, J. A., and Milne, L. J. R. (1987). Inhibition of phagocyte migration and spreading by spore diffusates of *Aspergillus fumigatus*. *J. Med. Vet. Mycol.* **55**, 1093–1099.

Robertson, M. D., Seaton, J. A., Raeburn, J. A., and Milne, L. J. R. (1987). Suppression of host defences by *Aspergillus Fumigatus*. *Thorax*. **42**, 19–25.

Rylander, R. (1987). The role of endotoxin for reactions after exposure to cotton dust. *Am. J. Ind. Med.* **12**, 678–697.

Sasada, M., Pabst, M. J., and Johnson, R. B. (1983). Activation of mouse peritoneal macrophage by lipopolysaccharide alters the kinetic parameters of the superoxide-producing NADPH oxidase. *J. Biol. Chem.* **258**, 9631–9635.

Sorenson, W. G., Shahan, T. A., Milanowski, T. A., and Lewis, D. M. Role of fungal spores in ODTS. *In* "Practical Applications in Health and Agriculture" (J. A. Dosman, H. H. McDuffie, S. A. Olenchock, K. M. Semchuk, and A. Senthilselvin, Eds.). Lewis Publishers, Chelsa, MI, in press.

Suzuki, K., Yamamoto, T., Sato, A., Murayama, T., Amitani, R., Yamamoto, K., and Kuze, F. (1983). Lipopolysaccharide primes human alveolar macrophage for enhanced release of superoxide anion and leukotriene B_4: Self limitations of the priming response with protein synthesis. *Am. J. Respir. Cell Mol. Biol.* **8**, 500–508.

Terashita, Z., Immura, Y., Nishikawa, K., and Sumida, A. (1985). Is platelet activating factor (PAF) a mediator of endotoxin shock? *Eur. J. Pharmacol.* **109**, 257–261.

Ward, P. A., and Johnson, K. J. (1986). Oxygen radicals and arachidonate metabolites in lung injury. *Ann. N.Y. Acad. Sci.* **456**, 26–33.