Concentration- and Time-dependent Upregulation and Release of the Cytokines MIP-2, KC, TNF, and MIP-1α in Rat Alveolar Macrophages by Fungal Spores Implicated in Airway Inflammation

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Inhalation of fungal spores has been shown to cause primary or secondary infection and respiratory inflammation and diseases such as allergic alveolitis, atopic asthma, and organic dust toxic syndrome, which are rarely reported in the absence of predisposing factors. Biochemical and molecular markers of inflammation were measured in rat bronchial alveolar lavage cells (> 95% macrophages) following stimulation with fungal spores isolated from pathogenic and nonpathogenic fungi that have been implicated in airway inflammation. The results of this study demonstrate that mRNA transcripts for the C-X-C branch of the PF4 superfamily are differentially upregulated over those of the C-C mediators in a time- and concentration-dependent manner. Macrophage inflammatory protein (MIP)-2 and KC were differentially upregulated over the acute phase inflammatory cytokines MIP-1α and tumor necrosis factor-α (TNF-α) in rat alveolar macrophages stimulated with fungal spores from *Aspergillus candidus*, *Aspergillus niger*, *Eurotium amstelodami*, and *Cladosporium cladosporioides*. Spores from *Aspergillus terreus* and *Penicillium spinulosum* failed to stimulate an increase of any cytokine mRNA, whereas those from *Aspergillus fumigatus* stimulated the upregulation of MIP-2, KC, TNF-α, and MIP-1α mRNAs. Over time, *A. fumigatus* stimulated increasing KC production until 24 h, when production levels increased slightly, then leveled off when measurements ceased at 36 h. Latex spheres stimulated modest amounts of MIP-2 and transforming growth factor-β only. These observations suggest that the inflammatory cytokines MIP-2 and KC may be involved in the inflammation arising from the inhalation of fungal spores in a time- and concentration-dependent manner. **Shahan, T. A., W. G. Sorenson, J. D. Paulauskis, R. Morey, and D. M. Lewis. 1998. Concentration- and time-dependent upregulation and release of the cytokines MIP-2, KC, TNF, and MIP-1α in rat alveolar macrophages by fungal spores implicated in airway inflammation. Am. J. Respir. Cell Mol. Biol. 18:435–440.**

Inhalation of fungi and fungal spores may cause infection and/or inflammation, which is dependent on the nature of the fungi as well as an individual's immune status (1, 2). Some of these opportunistic and nonpathogenic fungi are inhaled each day and are normally cleared from the airways without any symptoms. However, an increasing respiratory burden may result in respiratory inflammation (1). Fungal spore-mediated inflammation may be caused by either antibody-dependent or -independent mechanisms. Of the two, less is known about antibody-independent inflammation. Organic dust toxic syndrome (ODTS), an example of an antibody-independent pulmonary inflammation, is associated with exposure to high levels of organic dusts, especially organic dusts composed predominantly of fungal spores. ODTS is characterized by neutrophilic infiltration into the airways with systemic flu-like symptoms occurring 4–8 h after exposure (1, 2); however, the mechanism by which fungal spores or organic dust may induce pulmonary inflammation has not been defined. Alveolar macrophages (AMs) are the respiratory system's first line of defense and are known to contribute to inflammation by producing both lipid and protein mediators. It has been proposed that cytokines elicited from these immune effector cells mediate the inflammatory response

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Abbreviations: alveolar macrophage, AM; bronchial alveolar lavage, BAL; Iscove's modified Dulbecco's medium, IMDM; lipopolysaccharide, LPS; macrophage inflammatory protein, MIP; organic dust toxic syndrome, ODTS; transforming growth factor, TGF; tumor necrosis factor-α, TNF-α.

following the inhalation of fungal spores; however, limited cytokine data are available (3–5). We hypothesize that the spores contribute to the inflammatory response by inducing the production of inflammatory cytokines by AMs. We have evaluated this hypothesis by studying the response of AMs to spores from a variety of fungal species.

The proinflammatory cytokines macrophage inflammatory protein (MIP)-2 and KC are members of the C-X-C chemokine family (6, 7). Other members of this family include interleukin (IL)-8 and GROα/β/γ, which have been described almost exclusively from humans (8). It has been suggested that KC and MIP-2 are the rat physiologic equivalents of IL-8 and GRO (9). There is no standardized terminology for these substances between species. KC and MIP-2 are also known as the cytokine-induced neutrophil chemoattractants (CINC)/gro (10) and CINC-3/gro (11), respectively.

Acute phase inflammatory mediator MIP-1 is a member of the heparin-binding C-C chemokine family of proteins that are synthesized by macrophages and by many other cell types, and has significant inflammatory activities (12–16). Injection of MIP-1α into the footpads of mice was shown to cause localized inflammation with massive neutrophilic infiltration (17). Tumor necrosis factor-α (TNF-α) is another acute phase inflammatory agent that can initiate the coordinate upregulation of cytokines necessary for the maintenance and propagation of the inflammatory process (18). Transforming growth factor (TGF)-β family members are synthesized by numerous cell types, including macrophages; demonstrate chemoattractive activity for monocytes and macrophages; and are commonly found in areas of chronic inflammation (19). We suspected that TGF-β would have an effect early in the inflammatory process by attracting more monocytes and macrophages to the lung to obtain a more pronounced effect.

To determine whether these selected cytokines play an important role in fungal spore-mediated inflammation, cytokine-specific mRNAs were directly measured for MIP-2, KC, TNF-α, and MIP-1α by Northern blot analysis, and for TNF by a specific bioassay. TGF-β was analyzed only via Northern blot analysis. Previous studies in our laboratory to elucidate a mechanism for fungal spore-mediated inflammation had shown that bronchoalveolar lavage (BAL) cells stimulated with fungal spores produce neutrophil chemotactic substances, but the only one identified to date was leukotriene B_4 (5). Additional studies in our laboratory showed that fungal spores failed to stimulate BAL cells to produce and/or release IL-1 (20), but spores of the same species stimulated superoxide anion production in BAL cells. The magnitude of this response was species-dependent (21), i.e., spores from different species of fungi activated macrophages to different degrees. Lipopolysaccharide (LPS) failed to activate BAL cells to produce superoxide anion but was found to prime these cells for enhanced superoxide anion production following further stimulation with fungal spores (21). The objective of the present study was to continue our study of the mechanism(s) for fungal spore-mediated inflammation and to contribute to our understanding of the immunologic mediators and conditions that may function to initiate and propagate the inflammatory response.

Materials and Methods

Fungal Spores

Aspergillus fumigatus Fres., *Aspergillus niger* van Tieghem, *Aspergillus terreus* Thom, *Aspergillus candidus* Link ex Link, *Eurotium amstelodami* Mangin (=*Aspergillus amstelodami* [Mangin] Thom & Church), *Cladosporium cladosporioides* (Fres.) de Vries, and *Penicillium spinulosum* Thom were isolated from dust samples associated with episodes of ODTS as described previously (20) and were maintained by lyophilization. Spores were killed by autoclaving in endotoxin-free phosphate-buffered saline at 121°C, 15 PSI for 30 min and washed 5 times with the same buffer.

Bronchial Alveolar Lavage Cell Isolation

BAL cells were harvested from male rats (strain CD; Hilltop Laboratory Animals, Scottsdale, PA) by BAL using Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, as previously described (21). The cells from several animals were pooled and washed with HBSS without Ca^{2+} and Mg^{2+}. Cell densities were measured by a Coulter counter with channelizer (Coulter Electronic, Miami, FL) before plating. Macrophages comprised 95–98% of the BAL cell population. All media used, as well as representative samples of BAL fluid and spore preparations, were tested for endotoxin using Limulus amebocyte lysate analysis (Kinetic QCL; Biowhittaker, Inc., Walkerville, MD).

Northern Blot Analysis

BAL cell density was adjusted to 1×10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM)–15% horse serum, and 5 ml were placed into 100-cm-diameter tissue culture dishes and incubated for 60 min. Spores, LPS (positive control, *Escherichia coli* strain 0111:B4; DIFCO, Detroit, MI) or latex spheres (negative control, 2 μM; Sigma Chemical Co., St. Louis, MO) were added at the appropriate concentrations or densities for the times indicated.

Total cellular RNA was isolated from rat BAL cells using a modified procedure of Chirgwin (22). Briefly, cells were lysed with 4.0 M guanidine isothiocyanate solution and centrifuged for 18–20 h (18°C, 35,000 × *g*). The RNA was separated on a 1/18% agarose/formaldehyde gel (10 μg/lane). The RNA was transferred to a positively charged nylon membrane (Schleicher and Schuell, Keene, NH) and ultraviolet (UV) cross-linked at 1,200 joules/m. To detect gel loading consistency, bands were stained with EtBr and 18S and 21S bands were visualized with UV light and photo-documented.

Most cDNA inserts were prepared by incorporating biotinylated dUTP using the procedure of Lo and colleagues (23). All probes were polymerase chain reaction (PCR)-generated, rat-specific probes except MIP-1α, where a 30-mer antisense oligonucleotide (5′-GAA GCA GCA GGC AGT CGG GGT GTC AGC TCC-3′) was labeled with biotinylated dATP by terminal deoxynucleotidyl transferase using a protocol from Life Technologies (Grand Island, NY). MIP-2 and KC cDNA inserts were in plasmid pCR 1000 (Invitrogen, San Diego, CA); TNF-α and TGF-β probes were in pBR322 obtained from American Type Culture Collection (Rockville, MD). Briefly, plasmid DNA

(20–30 ng) was denatured for 10 min at 100°C, then quickly cooled on ice. Universal pUC/M13 amplification primers or SP6/T7-amplification primers were used where suitable. Final concentrations of reactants were: 50 mM KCl; 10 mM Tris-HCl, pH 9.0; 1.5 mM MgCl; 0.01% gelatin; 15 pM of each primer; 0.2 mM each of dATP, dGTP, and dCTP; and 0.135 mM dTTP with 0.065 mM dUTP-7 biotin (Sigma). To this solution, 5 U of Taq DNA polymerase was added to siliconized PCR tubes and amplification consisted of 35 cycles; 1 min denature at 94°C, 2 min anneal at 55°C, and 2 min extension at 74°C. The product was resolved on a 1.0% Tris boric acid-EDTA agarose gel and visualized with a UV transilluminator.

Northern blot membranes were blocked with Church's buffer (0.5 M $NaPO_4$, 1 mM EDTA, 7% sodium dodecyl sulfate, 150 μg/ml tRNA) for 3 h and incubated with approximately 45 ng labeled probe/100 cm^2. Membranes were washed in 0.1× saline sodium citrate (SSC) buffer for 15 min at room temperature and 3 times for 15 min each at 40°C before the membranes were incubated with streptavidin-alkaline phosphatase (Schleicher and Schuell) for 10 min. Membranes were then placed on luminol saturated filter paper (Schleicher and Schuell) before autoradiography. Band density on X-ray film was analyzed using a Millipore densitometer (Millipore Corp., Bedford, MA). Images of the autoradiographs were acquired using a Dage-MTI black-and-white CCD camera (Model CCD-100; Michigan City, MI) with tungsten backlighting. Digital images were downloaded into Photoshop (Adobe Systems, Salinas, CA), and grouped images were printed from a dye transfer sublimation thermal printer (Seiko Color Point PS 830; San Jose, CA), labeled, and photographed.

Tumor Necrosis Factor Bioassay

TNF release was analyzed using the TNF-specific bioassay described by Shahan and associates (24), with minor modifications. BAL cells were exposed to various treatments at different concentrations and lengths of time, as indicated previously, and conditioned media were collected and frozen at −80°C until time of assay. Briefly, 200 μl of cell suspension was added to each well to yield a final L-929 cell density of 2×10^4 cells/well and incubated (24 h at 37°C). The media were replaced with 100 μl of IMDM-15 containing 2.0 μg/ml actinomycin D and 50 μl of either a recombinant murine tumor necrosis factor (rmTNF-α) standard solution (R&D Systems, Minneapolis, MN) or an unknown sample, incubated (24 h at 37°C), and replaced with 150 μl of IMDM-15% horse serum solution containing 10% Alamar blue (Alamar Biosciences, Westbrook, OH). The cells were incubated with the indicator for 12 h, at which time the plates were read on a fluorescence concentration analyzer for 96-well plates (IDEXX Laboratories, Westbrook, ME) using a 545–575-nm band pass filter with the gain set at 1.

Statistical Analysis

TNF data were analyzed using Sigmaplot (Jandel Scientific Software, San Rafael, CA) and data were expressed as mean ± SD. Standard concentration curves were prepared from relative fluorescence units from known concentrations of rmTNF-α. Concentrations of TNF in unknowns were determined by linear regression from standards on each plate. The lowest limit of detection was defined as the lowest concentration of cytokine whose mean ± SD does not overlap with the mean ± SD of the negative control.

Results

Northern Blot Analysis of Total Cellular Cytokine RNA

Freshly lavaged rat BAL cells were exposed to fungal spores for up to 24 h before RNA was extracted and cytokine mRNAs were measured by Northern blot analysis.

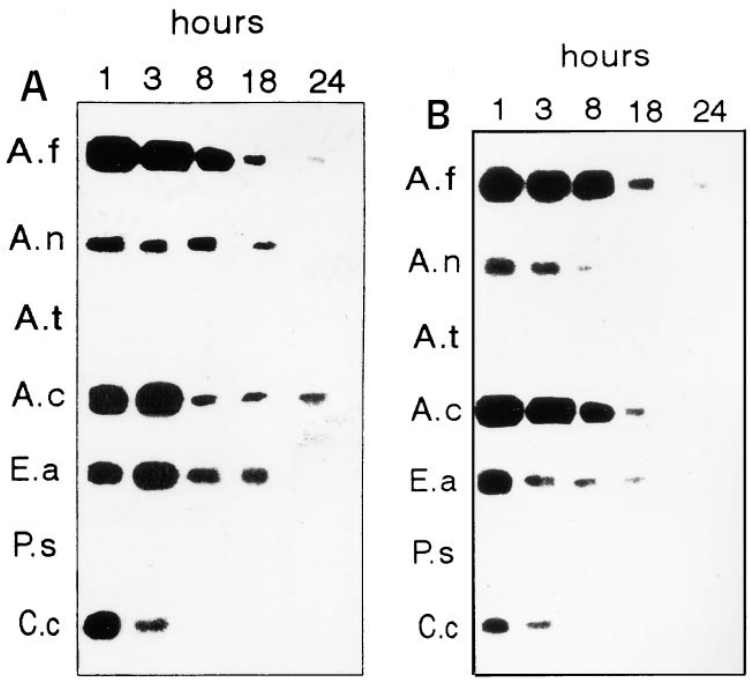

Figure 1. Kinetics of (*A*) MIP-2 mRNA and (*B*) KC expression in rat BAL cells in response to fungal spores. Rat BAL cells were exposed to fungal spores (1×10^8/ml) for 1, 3, 8, 18, or 24 h. Total RNA (10 μg) was resolved on a 1/18% agarose/formaldehyde gel, blotted to a nylon membrane, and probed with PCR-generated MIP-2 and KC probe. A.c = *A. candidus*, A.f = *A. fumigatus*, A.n = *A. niger*, A.t = *A. terreus*, E.a = *E. amstelodami*, P.s = *P. spinulosum*, C.c = *C. cladosporioides.*

Measurements of MIP-2 and KC showed similar but not identical patterns of induction. MIP-2 and KC mRNA transcripts were rapidly upregulated in BAL cells in response to spores from *A. fumigatus*, *A. niger*, *A. candidus*, *C. cladosporioides*, and *E. amsteladami*. Within 1 h, spores from each species stimulated peak amounts of MIP-2-specific mRNA, except for *A. candidus* and *E. amsteladami* which peaked between 1 and 3 h (Figure 1A). Within 1 h, spores from each species stimulated peak amounts of KC-specific mRNA and persisted from 8–24 h depending on the species tested (Figures 1A and 1B). *A. terreus* and *P. spinulosum* failed to upregulate either cytokine. TNF-α and MIP-1 mRNA were upregulated by *A. fumigatus* spores and LPS only (Figures 2A and 2B). LPS caused the greatest upregulation of all cytokine mRNA tested except TGF-β. All cytokine mRNAs studied except TGF-β were upregulated by 3 h in response to LPS. Pretreating BAL cells with LPS followed by treatment with fungal spores stimulated no further increase in cytokine-specific mRNA over that induced by LPS alone. Latex spheres caused only minor increases in MIP-2 and TGF-β mRNA but no change in KC. There was no change in specific mRNA concentrations in the cell-medium control (data not shown).

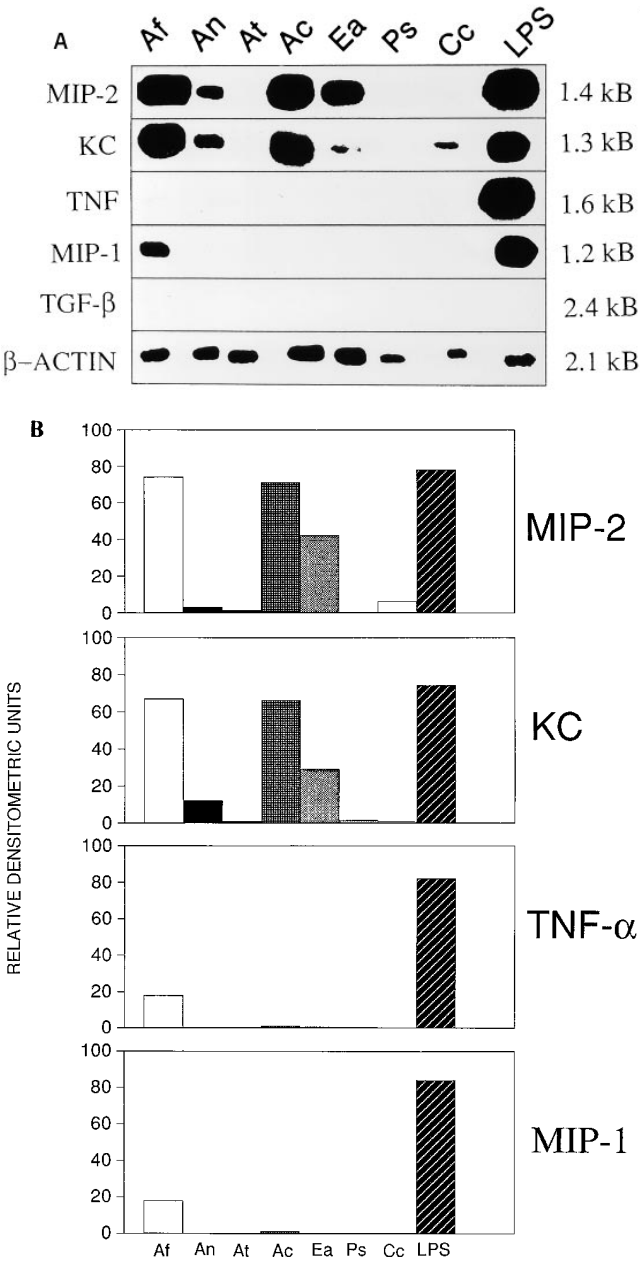

Figure 2. (*A*) Expression of MIP-2, KC, TNF-α, MIP-1, and β-actin mRNA in rat BAL cells following a 3-h incubation with either fungal spores (1×10^8) or LPS (10 μg/ml). Rat BAL cells were exposed to a stimulant for 3 h and total RNA (10 μg) was resolved on a 1/18% agarose/formaldehyde gel. RNA was blotted and probed with biotinylated MIP-2, KC, TNF-α, MIP-1, and β-actin probes. (*B*) Densitometric measurements of mRNA from (*A*). Data were obtained using a densitometer, were normalized on the basis of hybridized β-actin mRNA, and are expressed as arbitrary normalized densitometric units. A.c = *A. candidus*, A.f = *A. fumigatus*, A.n = *A. niger*, A.t = *A. terreus*, E.a = *E. amstelodami*, P.s = *P. spinulosum*, C.c = *C. cladosporioides*.

Tumor Necrosis Bioassay

BAL cells were stimulated with each of the seven fungi and TNF-α was measured using the Alamar blue L-929-A12 TNF bioassay as described by Shahan and associates (24). *A. fumigatus* stimulated BAL cells to produce and release TNF-α in a concentration-dependent fashion, whereas the other six fungi stimulated considerably less TNF-α (Table 1). *A. fumigatus* spores (1×10^3) stimulated rat BAL cells to release nearly as much TNF-α as was produced when the cells were treated with 100- and 100,000-fold higher numbers of spores. The other fungi at similar concentrations stimulated BAL cells to release 80–158 ± 5–13 pg/ml TNF (Table 1), and the positive control (1 μg/ml LPS) stimulated BAL cells to release 830 ± 56 pg/ml TNF. To determine whether LPS could prime BAL cells for enhanced TNF-α release following further stimulation with *A. fumigatus* spores, as described by Shahan and colleagues (21), a multiple analysis experiment was performed. LPS and fungal spores were tested alone and in combination at different concentrations of spores (10^3, 10^5, 10^7, 10^8, and 10^9 spores/ml) and LPS (1, 10, 100, and 1,000 ng/ml). No priming was seen at any concentration or com-

TABLE 1

*The density-dependent effect of fungal spores on the release of TNF-α from BAL cells at 24 h after exposure**

Species	10^3 Spores	10^5 Spores	10^8 Spores
A. fumigatus	597 ± 38†	758 ± 53	860 ± 43
A. niger	5 ± 4.3	12 ± 1.1	27 ± 5.2
A. terreus	14 ± 5.1	22 ± 4.4	33 ± 9.1
A. candidus	18 ± 3.4	31 ± 2.2	37 ± 3.3
E. amstelodami	14 ± 5.2	19 ± 3.3	24 ± 5.1
P. spinulosum	20 ± 2.2	25 ± 4.5	28 ± 2.2
C. cladosporioides	24 ± 3.3	34 ± 4.2	29 ± 3.7

* Fungal spores (1×10^3, 1×10^5, or 1×10^8) were incubated with 2×10^4 rat BAL cells for 18 h and conditioned media were assayed using the Alamar blue viability assay.

† pg TNF/ml; 1 μg/ml LPS (positive control) stimulated 830 ± 56 pg/ml.

binations of stimulants. There was no augmentation of TNF release above LPS levels by any spore concentration. Latex spheres or medium failed to stimulate TNF production (data not shown). Treatment of *A. fumigatus* conditioned medium with anti-TNF-α antibody (R&D) fully ablated any TNF activity as determined by the L-929 bioassay (data not shown).

Discussion

We have demonstrated that the C-X-C cytokines MIP-2 and KC are produced by BAL cells exposed to fungal spores. Our findings also suggest that mRNAs for the C-X-C cytokines MIP-2 and KC are rapidly and markedly induced in rat BAL cells by all fungal spores tested, except *A. terreus* and *P. spinulosum*, in a concentration-dependent manner. MIP-2 mRNA levels peaked at 1 h after exposure for *A. fumigatus*, *A. niger*, and *C. cladosporioides* and steadily decreased; however, in response to *A. candidus* and *E. amstelodami*, levels peaked between 1 and 3 h after exposure and steadily decreased (Figure 1A). All KC mRNA levels peaked at 1 h after exposure and steadily decreased (Figure 2). In addition to transcriptional activation of these genes, this exceedingly rapid increase in mRNA is consistent with mRNA stabilization. Both MIP-2 and KC have reiterated copies of the AU_NA motif on the 3′ untranslated region that has been shown to decrease the half-life of mRNA containing this motif when occupied by an mRNA binding protein (adenosine–uridine binding protein) (25). MIP-1α mRNA decays within 1 h in an unstimulated macrophage. LPS treatment of this cell line resulted in a concentration-dependent increase in MIP-1α steady-state mRNA expression which was partially the result of mRNA stabilization, since the half-life increased to over 6 h (16).

TNF-α and MIP-1 mRNA were upregulated in response to *A. fumigatus* and LPS only. Latex spheres caused only minor upregulation of MIP-2 mRNA and little or no upregulation of KC mRNA, which would suggest that the upregulation of MIP-2 and KC mRNA in response to most fungal spores was much different from particle effects seen with latex spheres.

Factors affecting fungal spore-induced inflammation and cytokine production have not been explored. In this study we have demonstrated that fungal species, spore concentration, and time each have an effect on the production and release of inflammatory cytokines. Although we have no data on clearance of spores of these fungi from the lung, it is likely that differential clearance rates could have an important effect on the prolongation of airway inflammation. Inhalation of a common airborne species such as *C. cladosporioides* may not cause any inflammation even at very high concentrations; however, species such as *A. fumigatus*, *A. candidus*, and *E. amstelodami* may cause airway inflammation at relatively low spore densities.

Little is known about the structural and biochemical nature of fungal spores that causes the differential upregulation of cytokines in BAL cells. Fungal cell walls are rigid structures composed of several different components, which include polysaccharides, proteins, lipids, pigments, and organic salts. Of these, polysaccharides commonly comprise more than 80% of the cell-wall dry weight, and β-glucans represent major structural components in the cell walls of many fungi (26). β-glucans are thought to be the immunologically active component in fungal cell walls and appear to exert much of their immune stimulatory effect via macrophage activation (27).

The potential of MIP-2 and KC to contribute to neutrophil chemotaxis and activation during pulmonary inflammation is suggested by a number of observations using both *in vitro* and *in vivo* studies in rats (12, 13). First, the KC gene product was shown to be chemotactic for neutrophils; however, it was not the exclusive mediator of polymorphonuclear neutrophil (PMN) activation during LPS-induced pulmonary inflammation (12). Second, using a rat model for LPS-induced acute lung inflammation, Xing and coworkers (28) measured several acute phase inflammatory cytokines and chemokines using Northern blot analysis of separate AM and PMN. Within one hour after challenge with LPS, MIP-2 levels in AM were higher than in PMN. Six hours later, the initial AM influx had been replaced with PMN. Most of the PMN that were gathered around the bronchiole and vasculature were shown to contain MIP-2 mRNA by the *in situ* hybridization technique. The importance of KC and MIP-2 in lung inflammation was demonstrated by the ablation of neutrophil accumulation following LPS challenge in the rat lung by anti-KC (29) and anti-MIP-2 (12, 30) antibodies. These data suggest that MIP-2/KC induction may be contributing to the acute inflammation induced by LPS.

Previous studies from our laboratory have shown that fungal spores differentially activate BAL cells as detected by measurement of superoxide anions (21). In this article we describe the differential upregulation of chemokine mRNA and proteins by the same fungal spores. To further understand the differential activation of BAL cells and their subsequent liberation of inflammatory mediators, protein tyrosine/serine/threonine phosphorylation patterns are being compiled. Using this unique model of cytokine induction, preliminary data indicate that tyrosine phosphorylation of specific proteins differ with the fungal species, especially those capable of stimulating differential upregulation.

In summary, the present study shows that MIP-2 and KC are upregulated in rat BAL cells in response to the spores of all fungal species studied except *A. terreus* and *P. spinulosum*. Only *A. fumigatus* stimulated TNF-α production. None of the different species studied stimulated TGF-β. Latex spheres stimulated negligible upregulation of TGF-β and MIP-2 only, which suggests that most species of fungal spores tested are more immunomodulatory than inert particles. These data indicate that the species from which the spore came may be the most important factor in spore-mediated pulmonary inflammation. The data further lend support for the idea that the inhalation of spores may cause pulmonary inflammation by the nearly exclusive production of C-X-C cytokines.

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