Studies from our laboratory (Shahan, T. A., Sorenson, W. G., and Lewis, D. M. (1994) *Environ. Res.* 67, 98–104) demonstrated that spores from different fungal species differentially activate rat alveolar macrophages as detected by the measurement of superoxide anion and cytokine production (Shahan, T. A., Siegel, P. D., Sorenson, W. G., Kuschner, W. G., and Lewis, D. M. (1998) *Am. J. Respir. Cell Mol. Biol.* 18, 435–441). Spores from *Aspergillus candidus* stimulated production of the highest levels of superoxide anion (5.2 nmol/1.0 × 10^6 alveolar macrophages (AMs)/30 min), followed by those from *A. niger* (2.4 nmol/1.0 × 10^6 AMs/30 min) and *Eurotium amstelodami* (0.4 nmol/1.0 × 10^6 AMs/30 min). The mechanism of this differential activation was studied. Our data demonstrate that the tyrosine kinases p56^Hck^, p72^Syk^, p77^Btk^, p65^cErk^, p56^Lck^, and p59^Fyn^ were specifically activated in response to spores from *A. candidus*, whereas spores from either *A. niger* or *E. amstelodami* activated p56^Hck^, p72^Syk^, and p77^Btk^. Kinetic analysis of specific tyrosine kinases demonstrated that p56^Hck^, p72^Syk^, and p77^Btk^ were activated faster and to a greater extent by spores of *A. candidus* as compared with spores from *E. amstelodami*. These data suggest a relationship between reactive oxygen species and tyrosine kinase activation. Treatment of AMs with H_2O_2 (1 mM) caused the activation of p72^Syk^ only, whereas treatment with superoxide dismutase and catalase before treatment with the spores had no effect on tyrosine kinase activation. Incubation with NADPH oxidase inhibitors inhibited both superoxide anion production and the activation of p56^Hck^, p72^Syk^, and p77^Btk^ in response to fungal spores. These data indicate that endogenous reactive oxygen species are necessary for the activation of p56^Hck^, p72^Syk^, and p77^Btk^ by spores; they also indicate that some species of spores are capable of activating tyrosine kinases independent of superoxide anion.

Immune cell types including polymorphonuclear leukocytes (PMNs)^1^ and macrophages respond to foreign matter and microorganisms by the production and release of immune mediators, antimicrobial agents, reactive oxygen species (ROS), and proteolytic enzymes (1, 2). Superoxide anion O_2^- and its dismutated product, hydrogen peroxide (H_2O_2), are powerful oxidants. ROS are potent antimicrobial agents (3) and, under conditions of high microbial load, cause tissue damage (4).

Studies by Sorenson et al. (5) demonstrated that fungal spores from defined species can be readily isolated from materials associated with outbreaks of organic matter-induced lung disease. Other studies showed that spores from *Aspergillus candidus* cause the production of much higher levels of O_2^- from alveolar macrophages (AMs) than spores from *Eurotium amstelodami* (6). The same spores were also demonstrated to differentially initiate the production of inflammatory cytokines (7). Since O_2^- is produced immediately in response to fungal spore exposure, we hypothesized that ROS production in response to fungal spores regulates signal transduction pathways that mediate inflammatory processes.

Apart from the antimicrobial function of ROS, they also function as intracellular signaling molecules. ROS are small molecules that favor their role in the intracellular signaling mechanism (8, 9). Eukaryotic cells possess enzymes capable of rapid and precise regulation of intracellular ROS levels (9). Additional evidence suggesting a role for ROS as signal transduction mediators includes their ability to activate the nuclear transcription factor NF-κB (10) as well as the demonstration that nitric oxide regulates neurotransmission and cell-mediated immune responses (11).

ROS have been demonstrated to be important in the regulation of protein-tyrosine kinase activation. Schiene et al. (12) showed that treatment of PMNs with H_2O_2 activates the cytoplasmic tyrosine kinases p56^Lck^, p72^Syk^, p59^Fyn^, and Zap70. In addition, ROS have also been shown to regulate extracellular signal-regulated kinases 1 and 2 in PMNs (10).

ROS are produced through a multicomponent enzyme, NADPH oxidase, which is located in the cell membrane of most leukocytes; however, most cells produce ROS as a side product of electron transport (14). Assembly of the oxidase in response to a stimulus imparts the ability to transfer one electron from NADPH to molecular oxygen to make O_2^-. Dismutation or reaction with other molecules can yield hydrogen peroxide, hydroxyl radical, peroxynitrate, and hypochlorous acid (15).

To investigate the effect of NADPH oxidase and ROS on tyrosine kinase activation, Brumell and Grinstein (16) activated the enzyme by incubating PMNs with guanosine-5' O-(3-thiophosphate) and NADPH. Activating the NADPH oxidase in

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‡The abbreviations used are: PMNs, polymorphonuclear leukocytes; ROS, reactive oxygen species; AM, alveolar macrophage; PAGE, polyacrylamide gel electrophoresis; WCLs, whole cell lysates; mAb, monoclonal antibody; NAC, N-acetylcysteine; MOPS, 4-morpholinepropanesulfonic acid; PVDF, polyvinylidene difluoride.
this manner eliminates receptor-operated phosphorylation, which could activate signal transduction pathways other than those directly linked to O₂⁻ production. Their data showed that p56Lck, p72Syk, and p77Btk were activated, and they concluded that endogenous ROS are able to regulate tyrosine kinase production.

In preliminary studies, we observed that AM treatment with fungal spores from different species stimulated O₂⁻ production with a concomitant increase in tyrosine phosphorylation of numerous proteins. With the demonstrated control that fungal spores have upon tyrosine phosphorylation, it follows that we would examine the effects of fungal spores and O₂⁻ on the activation of specific tyrosine kinases in AMs. In this study, we demonstrate that spores from different fungal species differentially activate tyrosine kinases. The phosphorylation and activation of individual tyrosine kinases are demonstrated to be due to the production of endogenous ROS, as evidenced by the inhibition of their activation in the presence of ROS scavengers, and unique molecular determinants on fungal spores. The ultimate goal of this research is to explain the mechanism that regulates the differential cellular responses (ROS production and cytokine production) to fungal spores.

**EXPERIMENTAL PROCEDURES**

**Materials**—Protein A-Sepharose and Sepharose beads, bovine serum albumin, and acrylamide were purchased from Roche Molecular Biochemicals. [γ-32P]ATP was purchased from NEN Life Science Products. The Nitroplus transfer membrane was purchased from MSI Separations (Westport, MA). Antibodies to p55/56 [AV], catalog no. L05620, p56f [Hk], p59 [Hk] (F19720), p62 [Hk] (Y35320), p90f [Hk] (R23820), p56f [Hk] (L15620), p135 [Hk] (T20220), Zap70 (Z24820), and p77 [Hk] (B85020) were obtained from Transduction Laboratories (Lexington, KY). Antibodies to p72/HPS (AB1374) obtained from Chemicon International, Inc. (Temecula, CA). p145[IT] (14101A) from Pharmingen (San Diego, CA), and p59f [F] (PEPA4) from Accurate Chemical & Science Corp. (Westbury, NY). Phosphotyrosine-reactive antibody PY20 was obtained from Zymed Laboratories Inc. (South San Francisco, CA). All horseradish peroxidase-labeled secondary antibodies were obtained from Rockland Inc. (Gilbertsville, PA). Latex beads and all other chemicals were obtained from Sigma.

**Fungal Spores—Aspergillus niger**

Alveolar Macrophage Isolation—Cells were harvested from several male rats (Harlan Sprague-Dawley, Taconic Farms Inc., Germantown, NY) by bronchial alveolar lavage fluid as well as cell culture medium were tested for endotoxin by the Limulus amebocyte lysate test (Kinetic QCL, BioWhittaker, Inc., Walkersville, MD). Cell densities were determined by a Coulter counter with a channelizer (Coulter Electronics, Miami Lakes, FL) before plating. AMs comprised 94–98% of the total bronchial alveolar lavage cell population. Cells were harvested from several male rats (Harlan Sprague-Dawley, Taconic Farms Inc., Germantown, NY) by bronchial alveolar lavage fluid as well as cell culture medium were tested for endotoxin by the Limulus amebocyte lysate test (Kinetic QCL, BioWhittaker, Inc., Walkersville, MD).

Measurement of Superoxide Anion—Superoxide anion was measured as described previously (6). AMs (1.0 × 10⁶ cells) were incubated with fungal spores (1 × 10⁷) for 30 min at 37°C or other agents as described below in the presence of cytochrome c. Superoxide anion released from AMs was measured by analysis of cytochrome c with or without superoxide dismutase as described under “Experimental Procedures.” Spores from A. candidus caused the highest levels of O₂⁻ with mean values of 4.9 nmol/l × 10⁶ cells/30 min, followed by A. niger with 2.4 nmol/
and H₂O₂ were produced by incubation of xanthine. This was assayed by measuring the reduction of cytochrome c (1.2 μM) with spectrophotometry at 550 nm. Data represent means ± S.E. (n = 10) for each measurement. Cell-only control = 0.9 ± 0.2 nmol/2.5 × 10⁶ AMs/30 min. *, p < 0.05 when comparing agents with the AMs-alone control.

Fungal Spores Differentially Activate Tyrosine Kinases in AMs—Phosphorylation of tyrosine residues has been demonstrated to be an important determinant in the regulation of cellular functions. To examine the effect of fungal spores on protein tyrosine phosphorylation, WCLs from spore-treated AMs were separated by SDS-PAGE and analyzed by Western blotting using the phosphotyrosine-reactive mAb (1:3500), followed by detection with ECL. AMs were incubated alone in lysis buffer, and WCLs were separated by SDS-PAGE, followed by immunoprecipitation with specific tyrosine kinase-reactive mAbs and probed with a horseradish peroxidase-labeled phosphotyrosine-specific secondary antibody and detected with ECL. The assay was also performed with rabbit nonimmune serum as a control and failed to detect any proteins. The data are representative samples of experiments performed on three different occasions.

Second, we examined the effect of fungal spores on the phosphorylation of specific tyrosine kinases in AMs following exposure to fungal spores. This was done by immunoprecipitation of the AM WCLs with specific tyrosine kinase-reactive mAbs without prior treatment or following incubation with either A. candidus (Ac), A. niger (An), or E. amstelodami (Ea); latex beads (LB); or cells alone (not treated (NT)). The data are representative samples of experiments performed on three different occasions.

Fungal Spores Differently Induce Tyrosine Phosphorylation in AMs—Phosphorylation of tyrosine residues has been demonstrated to be an important determinant in the regulation of cellular functions. To examine the effect of fungal spores on protein tyrosine phosphorylation, WCLs from spore-treated AMs were separated by SDS-PAGE and analyzed by Western blotting using the phosphotyrosine-reactive mAb PY20. At 5 min post-exposure, the level of phosphorylation was 12.3 ± 0.7 nmol of O₂⁻/10⁶ cells/30 min. As a negative control, latex beads stimulated AMs to produce 1.1 ± 0.3 nmol of O₂⁻/10⁶ cells/30 min, which was not different from control levels.

Identification of specific tyrosine kinases present in alveolar macrophages. Rat AMs were collected as described under “Experimental Procedures.” Cells (1 × 10⁷/ml) were incubated with either A. candidus (Ac), A. niger (An), or E. amstelodami (Ea); latex beads (LB); or cells alone (not treated (NT)). The data are representative samples of experiments performed on three different occasions.
Fungal Spores Differentially Activate Tyrosine Kinases

Kinetic Analysis of Specific Tyrosine Kinase Activation in Response to Spores from Different Fungal Species—Of those tyrosine kinases that were found to be expressed and activated, we specifically studied the kinetic activation of p56Hck, p72Syk, and p77Btk in response to spores from either A. candidus or E. amstelodami. To determine the effect of ROS scavengers and the NADPH oxidase inhibitor on O₂⁻ production, AMs were treated with the inhibitors before the addition of fungal spores in the presence of cytochrome c. All three agents inhibited O₂⁻ production as measured by cytochrome c reduction (data not shown).

It was suggested that ROS may be directly capable of activating these tyrosine kinases. To test this possibility, we isolated individual tyrosine kinases by immunoprecipitation, incubated each with either O₂⁻ or H₂O₂, and looked at the activation of each by Western blot analysis with the PY20 mAb. Our data indicate that neither O₂⁻ nor H₂O₂ was capable of directly activating the kinases p56Hck, p72Syk, and p77Btk, whereas p62Yes, p56Lck, and p59Fyn were activated by a different signaling pathway possibly due to a unique molecular determinant on spores from A. candidus. To determine the effect of ROS scavengers and the NADPH oxidase inhibitor on O₂⁻ production, AMs were treated with the inhibitors before the addition of fungal spores in the presence of cytochrome c. All three agents inhibited O₂⁻ production as measured by cytochrome c reduction (data not shown).

In this study, we investigated the signal transduction pathways that initiate macrophage activation in response to fungal spores that have been implicated in organic dust-induced lung inflammation. Our data demonstrate that fungal spores from different species differentially activate macrophages. It can be envisioned that the ability of microorganisms to evade immunological detection imparts additional pathogenicity. It is unknown whether these capabilities are due to the absence or presence of a specific ligand-receptor interaction because the immunological determinants on these and most fungi are unknown. Because ROS production is one of the most immediate responses of cell activation and were demonstrated to act as signal transduction mediators in the activation of macrophages, we studied the ability of ROS from fungal spore-treated AMs to influence tyrosine kinase activation.

DISCUSSION

To study the effect of ROS on tyrosine kinase activation, we inhibited the ability of AMs to produce ROS by inhibiting NADPH oxidase with deoxy-o-glucose (125 mM), diphenyleneiodonium (1250 μM), or iodonium biphenyl (1250 μM) (19), the latter two of which are potent NADPH oxidase inhibitors. Treatment of AMs with these agents inhibited the activation of p56Hck, p72Syk, and p77Btk in response to spores from either A. candidus or E. amstelodami. In addition, phosphorylation of p62Yes, p56Lck, and p59Fyn was also unaffected by any of the three agents in response to spores from A. candidus (data not shown). In each case, O₂⁻ measurements were made using the protocol described under “Experimental Procedures.” There was no O₂⁻ produced by the AMs following treatment with inhibitors or fungal spores. These data indicate that the signal transduction pathway that regulates the activation of these tyrosine kinases in response to fungal spores as described above requires ROS.

To further study the role of ROS as a signal transduction molecule capable of activating tyrosine kinases in response to fungal spores, AMs were incubated with NAC, a powerful antioxidant, before the addition of fungal spores. Treatment of AMs with NAC inhibited the activation of p56Hck, p72Syk, and p77Btk in response to spores from E. amstelodami and A. candidus; however, NAC had no effect on the activation of p62Yes, p56Lck, or p59Fyn in response to spores from A. candidus. These data further demonstrate that endogenous ROS are responsible for the activation of p56Hck, p72Syk, and p77Btk, whereas p62Yes, p56Lck, and p59Fyn are activated by a different signaling pathway possibly due to a unique molecular determinant on spores from A. candidus.

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show that spores from \textit{E. amstelodami} cause the activation of p56Hck, p72Syk, and p77Btk, whereas spores from \textit{A. candidus} cause the activation of the latter plus p62Yes, p56Lck, and p59Fyn. The significance of these differences is not completely understood. The activation of p56Hck, p59Fgr, and p72Syk by the direct activation of the NADPH oxidase, bypassing cellular receptors, has been previously described by Brumell and Grinstein (16) and lends additional support to the idea that these kinases are activated through a mechanism involving this enzyme.

Spores from \textit{A. candidus} stimulated the rapid activation of p56Hck, p72Syk, and p77Btk in AMs. p56Hck, a member of the Src family of tyrosine kinases, has been shown to be expressed in macrophages and is known to be necessary for tumor necrosis
Fungal Spores Differentially Activate Tyrosine Kinases

Fig. 6. Effect of ROS on tyrosine kinase phosphorylation in response to spores from different fungal species. Rat AMs (1 × 10^6 cells) were collected as described under “Experimental Procedures.” A, AMs were incubated with either H_2O_2 or xanthine/xanthine oxidase (Xan/Xan) and with catalase (CAT), superoxide dismutase (SOD), or catalase + superoxide dismutase for 5 min at 37 °C. B, AMs were additionally incubated with spores (1 × 10^6) from either A. candidus (AC) or E. amstelodami (EA) and with deoxy-d-glucose (DDG, 125 mM), diphenyleneiodonium (DPI, 125 μM), or NAC (50 μM). Cells were then lysed, and individual tyrosine kinases (p56Lck, p72Syk, and p77Btk) were isolated by immunoprecipitation and separated by SDS-PAGE. Proteins were transferred to a PVDF membrane, probed with a horseradish peroxidase-labeled phosphotyrosine-reactive mAb (1:3500), and detected with ECL. The data are representative samples of experiments performed on three different occasions with AMs obtained at different times.

...factor production in response to lipopolysaccharide (24). The incubation of AMs with either diphenyleneiodonium or NAC decreased tumor necrosis factor production in response to fungal spores and lipopolysaccharide.\(^2\) p72Syk was also activated after incubation with fungal spores. p72Syk activation has been shown to be necessary for Fcγ (CD64)-mediated phagocytosis and is thought to be necessary for immunological responses requiring interleukin-5 (24). p77Btk, a member of the Tsk family, was also demonstrated to be specifically activated in response to these spores. Like the other two kinases, it has been shown to be expressed in neutrophils (25).

Spores from A. candidus alone caused the activation of p62Yeast, p56Lck, and p59Fyn. p62Yeast has been shown to be expressed in both monocytes and macrophages (26) and is believed to play a role in cytoskeletal rearrangement because it is commonly found associated with cytoskeletal proteins following treatment with chemoattractants (27). p56Lck has also been demonstrated to be expressed in macrophages (28, 29). Altogether, the combined research on these tyrosine kinases is limited, so little inference can be made regarding their direct involvement in the differential activation of AMs in response to fungal spores. Indirectly, p56Lck has been demonstrated to activate the nuclear transcription factor AP-1 in human immunodeficiency virus-infected macrophages (30). Additionally, it has also been shown to activate SP-1, SP-2, and NF-κB in U937 cells (31). It is for these reasons that we have initiated a study to investigate the ability of fungal spores to activate nuclear transcription factors.

To determine the role of ROS in the mechanism underlying the differential activation of both cells and tyrosine kinases by fungal spores, the activation of tyrosine kinases in response to either H_2O_2 or O_2^- was studied. Our data demonstrate that spores from both E. amstelodami and A. niger activate p56Lck, p72Syk, and p77Btk in AMs. In response to spores from A. candidus, the latter kinases were activated as well as p62Yeast, p56Lck, and p59Fyn, whereas AMs treated with either H_2O_2 or O_2^- activated p72Syk only. Our data suggest that ROS levels correlate with tyrosine kinase activation. Fig. 1 demonstrates that spores from A. candidus caused higher levels of O_2^- in AMs as compared with spores from E. amstelodami. Likewise, Fig. 5 demonstrates that spores from A. candidus activated tyrosine kinases in AMs more rapidly and to a greater degree than spores from E. amstelodami.

These data also indicate that extracellular ROS have little effect on tyrosine kinase activation; however, they indicate nothing about tyrosine kinase activation resulting from receptor-ligand binding activation of NADPH oxidase or endogenous ROS resulting from the latter. The fact that the NADPH oxidase inhibitors deoxy-d-glucose (125 mM), diphenyleneiodonium (1250 μM), and iodonium biphanyl (1250 μM) inhibit fungal spore-induced O_2^- production as well as tyrosine kinase activation indicates that it is likely that both cell activation and p56Lck, p72Syk, and p77Btk activation in response to fungal spores are regulated through NADPH oxidase. To test this hypothesis, AMs were incubated with NAC, a powerful antioxidant, before the addition of fungal spores to neutralize endogenous ROS. This treatment inhibited the activation of p56Lck and p77Btk in response to spores from both species, but not of p62Yeast, p53/p56Lck, or p59Fyn in response to spores from A. candidus. Even though the data implicate both the NADPH oxidase and O_2^- in the signaling pathway, the only definitive way to determine the role of NADPH oxidase is to use knockout models or PMNs from humans deficient in or lacking the NADPH oxidase (chronic granulomatous disease of childhood). We are making plans to do so. Because there are likely many other unidentified tyrosine kinases in AMs, neither this study nor any other study surveying specific tyrosine kinases can be absolutely complete. This is the first report to demonstrate a link between cell activation, O_2^- and specific tyrosine kinase activation in response to fungal spores. More specifically, our data indicate that fungal spores from different species activate tyrosine kinases by endogenous ROS as well as by unique molecular determinants on spores from each fungal species independent of ROS.

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