Mucorales Species Activation of a Serum Leukotactic Factor

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Previous studies have suggested that the focal accumulation of phagocytic leukocytes is an important feature of the host response in mucormycosis. To ascertain the basis for this influx of inflammatory cells, we evaluated the effect of members of the order Mucorales, including species from the genera Rhizopus, Absidia, and Mucor, on the chemotactic activity of normal human serum for neutrophils and monocytes. Both hyphae and spores produced concentration-dependent chemotaxigenesis in serum to a maximum level equivalent to that produced by zymosan activation of serum. Chemotactic activity was similar for live and heat-killed hyphae. No leukotactic activity was demonstrated in the absence of serum. The pretreatment of serum with anti-C3 antibody, heating at 56°C, or 0.01 M EDTA abolished the activity. The pretreatment of serum with 0.01 M ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid did not abolish the activity. These data provide evidence that the leukotactic activity of Mucorales species is generated through the alternative complement pathway.

Mucormycosis is a severe, invasive, fungal infection occurring almost exclusively among individuals with compromised host defenses (13). Although members of the order Mucorales are ubiquitous in the environment, including hospital air (14), these fungi do not commonly cause disease in humans. Within this order, the three genera most frequently pathogenic for humans are Rhizopus, Absidia, and Mucor. After the inhalation of spores onto nasal or respiratory mucous membranes, germination occurs, and hyphal invasion results in inflammation, vascular thrombosis, and tissue necrosis.

Little is known of the defense mechanisms that are active in preventing or limiting disease by this opportunistic pathogen. Two types of host defenses against spore germination and hyphal invasion have been identified: a normal serum factor (7, 17) and phagocytic leukocytes (1, 18). In animal studies, an early influx of neutrophils and later monocytes has been demonstrated in mucormycotic lesions (18), but the mechanism of this aggregation of cells has not been determined. Previous investigators have reported that neutrophil chemotactic factors are produced by Candida albicans (4) and others are activated in serum by Cryptococcus, Cocci- dioides, and Candida spp. (5, 8, 12, 15). In the present study, we report that members of the order Mucorales incubated in normal human serum generated a chemotactic factor for neutrophils and monocytes. Similar leukotactic activity was not produced when fungi were cultured in the absence of serum.

MATERIALS AND METHODS

Cultivation of fungi. Absidia corymbifera NRRL 2981 from soil, Rhizopus rhizopodiformis 1046 from an infected renal transplant patient, Rhizopus oryzae 519 from a human nasal septum lesion, R. oryzae 497 from a human skin lesion (courtesy of Irene Weitzman, Department of Health, New York, N.Y.) Rhizopus arrhizus ATCC 14050 from a fatal human infection, and Rhizomucor pusillus (formerly designated Mucor pusillus) ATCC 36606 from a cat brain were propagated on chocolate agar at 37°C for 24 h. To produce large masses of hyphae, small amounts of fungi from the chocolate agar were introduced into bottles containing 50 ml of tissue culture medium 199 (GIBCO Laboratories, Grand Island, N.Y.) and incubated for 24 h at 37°C. Stock strains were stored on chocolate agar plates at −80°C.

Spore and hyphae preparation. Spores were harvested by flooding agar plates with sterile saline, agitating the hyphae and sporangia with a needle, filtering the suspension through fine-mesh gauze, and washing them in medium 199. The enumeration of the spores was performed with a Neubauer grid, and appropriate concentrations were made in medium 199. Hyphae cultivated in medium 199 were harvested by centrifugation (1,000 x g, 10 min), washed, and quantitated by wet weight. Serial dilutions of the supernatants and media controls were assayed for leukocyte chemotactic activity.

Isolation and labeling of neutrophils. Venous blood from normal human volunteers was collected into heparinized syringes (5 to 10 U/ml). Erythrocytes were sedimented by mixing blood with a 1% dextran solution (1/3 vol/vol; Sigma Chemical Co., St. Louis, Mo.; molecular weight: 500,000) for 0.5 h followed by centrifugation of the leukocyte-rich supernatant at 200 x g for 10 min. After resuspension in Hanks balanced
salt solution (HBSS) with calcium and magnesium (GIBCO), the leukocytes were layered over Isolyph (Gallard/Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y.) in a 4.3 ratio (vol/vol) and centrifuged at 380 × g for 35 min for density gradient separation of mononuclear cells from neutrophils. Residual erythrocytes were removed from the suspension by hypotonic lysis, and the neutrophils in HBSS were incubated with 2 μCi of Na35CrO4 (New England Nuclear Corp., Boston, Mass.) in 0.9% saline per 106 cells with agitation for 1 h at 37°C. Labeled neutrophils were washed three times in cold HBSS, and a final resuspension was made in medium 199 containing 1% bovine serum albumin (U.S. Biochemical Corp., Cleveland, Ohio) at a cell concentration of 2 × 106/ml. Cell suspensions consistently contained less than 5% mononuclear cells and demonstrated greater than 95% viability by trypan blue dye exclusion.

Neutrophil chemotaxis. Modified Boyden blind-well chambers (Bio-Rad Laboratories, Richmond, Calif.) were loaded with chemotactic stimulus and control media. The only different media used were in the lower compartment, a double microfilter to separate the compartments, and 35Cr-labeled cells above. The lower of the two filters was a 3-μm Sartorius (Science Essentials, Anaheim, Calif.), and the upper was a 5-μm Nuclepore (Arthur H. Thomas, Co., Philadelphia, Pa.). After a 1-h incubation of the chambers at 37°C in 100% humidity with a 5% carbon dioxide atmosphere, the cell suspension was aspirated off, and the upper filter was discarded. The lower filter was rinsed three times in saline and assayed for chromium-51 activity in a gamma counter (Beckman 9000 system; Beckman Instruments, Inc., Fullerton, Calif.). In this assay, which is a modification of that of Gallin et al. (10), a linear accumulation of radioactivity into the lower filter was demonstrated between 15 min and 2 h of incubation with no increase in the level of activity in the lower compartment fluid. Due to the variability of isotope incorporation into neutrophils, related to the specific activity of 35Cr, chemotactic activity was expressed as corrected counts per minute (Cor cpm) in the lower filter by adjusting the observed cpm with the following formula: Cor cpm = (Observed cpm (lower filter) × 10,000/cpm/106 neutrophils. Each experiment was conducted at least three times on different days, and each condition was performed in triplicate. Appropriate media and positive controls were included in each experiment. Statistical analysis was performed with Student’s t-test.

Monocyte chemotaxis. Venous blood from normal human volunteers was collected into a plastic bottle containing heparin (5 to 10 U/ml) and mixed with 0.85% saline (1:3 [vol/vol]). The mixture was layered over Isolyph (8.3 [vol/vol]) and centrifuged at 400 × g for 45 min. The monocyte layer was collected from the supernatant, washed, and resuspended in medium 199 containing 2% bovine serum albumin at a concentration of 1 × 109/ml. Chemotaxis was performed by the method of Campbell, using blind-well chambers as in the neutrophil assay (3). In loading the chambers, a double filter was used, which was modified for monocyte studies by using a 0.45-μm Sartorius as the lower filter and a 5-μm Nuclepore as the upper filter. After a 2-h incubation at 37°C in 100% humidity, the two filters were carefully removed as a unit, fixed in paraformaldehyde, and stained with hematoxylin. The filters were mounted on glass slides, and all monocytes were enumerated in each of five high-powered microscope fields of the lower surface of the 5-μm filter. Duplicate chambers were used for each condition. The mean of all 10 determinations was then calculated, and chemotaxis was expressed as monocytes per high-powered microscope fields ± standard error of the mean.

Serum studies. Normal human serum pooled from five donors was obtained by allowing venous blood to clot for 1 h at 25°C and centrifuging for 20 min at 1,000 × g. Portions (5 ml) were stored at −80°C until used. Various concentrations of hyphae (10, 50, 100, 200, 300, and 500 mg/ml) or of spores (0.625 × 106, 1.25 × 106, 2.5 × 106, 5.0 × 106, and 10 × 106/ml) were incubated with serum at 37°C for 1 h in plastic test tubes on a rotating rack. Preliminary studies demonstrated that 250 mg of hyphae per ml produced maximal chemotactic activity, and this concentration was used in all subsequent experiments with serum. Hyphae were removed from the serum by centrifuging at 1,000 × g for 10 min, and the serum was heated at 56°C for 1 h before immediate use in the chemotaxis assay. Where indicated, hyphae were killed by autoclaving at 30 min at 120°C and 1 kg/cm2. Cultures of this material demonstrated no growth.

Chemotactically active control serum was prepared by washing zymosan (Sigma) three times in medium 199 and incubating it in serum at a concentration of 25 mg/ml for 1 h at 37°C. Zymosan was removed by centrifugation at 1,000 × g for 10 min, and the serum was heated at 56°C for 1 h before use. Zymosan-activated serum (ZAS) as the positive control and medium 199 as the negative control were included in each chemotaxis experiment.

To characterize the leukotactic activity we pretreated serum with 0.01 M EDTA, 0.01 M ethylene glycolbis(β-aminoethyl ether)-N,N-tetraacetic acid (EGTA), or heating at 56°C for 1 h before incubation with fungal hyphae. After the C3 and factor B contents of the pooled serum were determined (76 and 33 mg/dl, respectively), using radial immunodiffusion plates (Calbiochem-Behring Corp., La Jolla, Calif.), the equivalent of 110% of goat antihuman C3 or factor B antibody (Kent Laboratories, Ltd., Vancouver, B.C., Canada) was preincubated with a portion of serum for 1 h at 37°C. Hyphae were then incubated with anti-C3 or anti-factor B antibody-treated and control serum in parallel, and the serum was assayed for chemotactic activity after the removal of hyphae.

RESULTS

Human serum preincubated with A. corymbifera hyphae demonstrated dose-dependent chemotactic activity for neutrophils by the Boyden chamber radioassay (Fig. 1). Untreated normal serum was chemotactic for neutrophils in this assay, but a significant enhancement of chemotaxis was observed after the interaction of serum and fungal hyphae, with proportionately greater differences seen at higher concentrations of serum (Fig. 1). The level of activity of either 5 or 10% serum preincubated with A. corymbifera closely approximated that produced when serum was activated with zymosan under the same conditions. A significant reduction of chemotax-
FIG. 1. Chemotactic response of neutrophils to various concentrations of serum preincubated with A. corymbifera hyphae (○) or control medium (○) to a negative control (medium 199; Δ) and to a positive control (5% ZAS; ▲). Other symbols: *, P < 0.05; †, P < 0.005; ‡, P < 0.001. Vertical bars indicate standard error of the mean.

Chemotaxis was not observed when heat- or ethanol-killed, rather than live, hyphae were incubated with serum (data not shown).

Table 1 compares the leukotactic activity produced by six strains of Mucorales in the presence or absence of serum. Although there was rapid growth of hyphae in medium 199, there was no detectable chemotactic activity in these supernatants in the absence of serum. In other experiments, further dilutions of the culture medium supernatant between 1:2 to 1:32 and variation of incubation periods from 3 to 48 h failed to produce evidence of neutrophil chemotactic activity. In contrast, the addition of serum to suspensions of hyphae uniformly resulted in chemotaxis by all six strains, including members of Rhizopus, Mucor, and Absidia spp. Strains isolated from the environment, from infected human patients, and from an infected animal (one) produced similar serum activation.

To determine the optimal concentration of hyphae producing an enhancement of serum activity, various amounts of Rhizopus hyphae from 10 to 500 mg/ml were preincubated with fresh, pooled serum (Fig. 2). At both 1 and 5% serum, maximal chemotaxis by all six strains, including members of Rhizopus, Mucor, and Absidia spp. Strains isolated from the environment, from infected human patients, and from an infected animal (one) produced similar serum activation.

Table 1. Chemotactic activity generated by Mucorales species in the presence or absence of serum

<table>
<thead>
<tr>
<th>Species</th>
<th>Chemotactic activity (Cor cpm × 10^{-3} ± SEM)</th>
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<tbody>
<tr>
<td></td>
<td>In medium 199a</td>
</tr>
<tr>
<td>Control (no hyphae)</td>
<td>1.1 ± 0.05</td>
</tr>
<tr>
<td>R. arrhizus</td>
<td>1.1 ± 0.06</td>
</tr>
<tr>
<td>R. oryzae (497)</td>
<td>1.2 ± 0.03</td>
</tr>
<tr>
<td>R. oryzae (519)</td>
<td>1.0 ± 0.08</td>
</tr>
<tr>
<td>R. rhizopodiformis</td>
<td>0.9 ± 0.01</td>
</tr>
<tr>
<td>R. pusillus</td>
<td>1.2 ± 0.03</td>
</tr>
<tr>
<td>R. corymbifera</td>
<td>0.9 ± 0.02</td>
</tr>
</tbody>
</table>

* Assayed by placing 1:2 dilutions of hyphae culture supernatants in the lower compartment of the chemotactic chamber.

b Assayed by placing 5% human serum preincubated with hyphae in the lower compartment of the chemotactic chamber.

c P < 0.001.

d P < 0.005.

FIG. 2. Chemotactic response of neutrophils to 1% (○) or 5% (●) serum preincubated with various concentrations of Rhizopus hyphae to a negative control (medium 199; Δ) and to a positive control (5% ZAS; ▲).
with monocytes. Concentrations of serum between 2.5 and 20% were tested for their relative monocyte-attracting effect after incubation with Rhizopus hyphae, control medium, or zymosan (Fig. 3). The peak effect of zymosan-activated serum at a dilution of 10% is apparent, as is the enhanced chemotactic effect of serum preincubated with hyphae.

Because hyphal preparations contained a number of spores, we investigated the possibility that contaminating spores were producing the serum-activating effect attributed to Mucorales hyphae. Comparing the neutrophil chemotactic activity of serum preincubated with increasing concentrations of spores with the activity generated by 250 μg of hyphae per ml contaminated with \(5 \times 10^5\) spores per ml, it is evident that the activity produced by the number of contaminating spores was no greater than that of the base line (Fig. 4). Higher concentrations of spores in the range of \(5 \times 10^6\) to \(1 \times 10^7\)/ml resulted in substantial serum activation.

To examine the possibility that Mucorales chemotaxigenesis might result from complement activation (Table 2), we employed the pretreatment of serum with EDTA, EGTA, or heating at 56°C for 1 h. Zymosan, which generates chemotactic activity through the activation of the alternate complement pathway, was used as the positive control as in previous experiments. The incubation of either Mucorales hyphae or zymosan with untreated serum resulted in significantly increased neutrophil chemotactic activity. The enhanced serum leukotactic activity produced by Mucorales was absent after the pretreatment of serum with EDTA or heating at 56°C, but was not inhibited after preincubation with EGTA. Parallel results were demonstrated for zymosan-activated serum. Finally, we attempted to confirm the complement origin of the leukotactic activity by investigating the effect of anti-C3 antibody on the serum chemotaxigenesis (Table 3). The usual leukotactic activity observed after the incubation of serum with Mucorales hyphae or zymosan was not present in the presence of anti-C3 antibody. Similarly, the preincubation of serum with anti-factor B anti-

![FIG. 3. Chemotactic response of monocytes to various concentrations of serum preincubated with Rhizopus hyphae (○), medium 199 (△), or zymosan (▲).](http://iai.asm.org/)

![FIG. 4. Comparison of the chemotactic response of neutrophils to 5% serum preincubated with various concentrations of Rhizopus spores (○) or 250 μg of hyphae per ml containing \(0.5 \times 10^6\) spores per ml (●). Negative (medium 199; △) and positive (5% ZAS; ▲) controls are included.](http://iai.asm.org/)

<table>
<thead>
<tr>
<th>Serum pretreatment</th>
<th>Chemotactic activity (Cor cpmp × 10^{-3} ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal serum</td>
</tr>
<tr>
<td>None (control)</td>
<td>1.80 ± 0.07</td>
</tr>
<tr>
<td>Heat (56°C, 1 h)</td>
<td>1.92 ± 0.02</td>
</tr>
<tr>
<td>EDTA (0.01 M)</td>
<td>1.95 ± 0.03</td>
</tr>
<tr>
<td>EGTA (0.01 M)</td>
<td>2.13 ± 0.01</td>
</tr>
</tbody>
</table>

\(a P < 0.001\).
body inhibited the Mucorales- or zymosan-induced chemotactic activity (data not shown).

**DISCUSSION**

Tissue invasion by *Mucorales* is characterized histologically by an intense neutrophilic infiltration both in humans (19) and in animals (16, 18). Additional evidence for the importance of phagocytes in this fungal infection is provided by the known susceptibility to mucormycosis of patients with leukemia and neutropenia (13) and the observation of Diamond et al. of the capability of neutrophils to damage *Rhizopus* hyphae in vitro (6). Sheldon and Bauer demonstrated the rapidity of the local accumulation of leukocytes, using a rabbit model of mucormycosis (18). After the intradermal inoculation of the animals with *R. oryzae* spores, local leukocyte margination in blood vessels was observed within 5 min, and early tissue invasion was observed within 10 min.

Chemotactic factors produced by pathogens or generated in serum are thought to be important in attracting phagocytes to the site of microbial invasion (11, 21). Previous investigators have described neutrophil chemotactic factors activated in serum by *Candida* species, *Coccidioides immitis*, and encapsulated and non-encapsulated *Cryptococcus neoformans* (5, 8, 12, 15). Chemotaxis by *Candida* sp. was directly proportional to the surface area of the fungi exposed to serum (5). Under the proper conditions, *C. albicans* also may produce chemotactic activity for guinea pig neutrophils in the absence of serum (4). Our experiments demonstrated the activation of a leukocyte chemotactic factor when *Mucorales* spores or hyphae were incubated with human serum in vitro. We were unable to identify chemotactic activity produced by *Mucorales* in the absence of serum. In our studies, rapid growth of *Mucorales* occurred in medium 199, and varying the period of incubation or the concentration of hyphae supernatant produced no evidence of leukotactic activity.

The serum-activating potential was not limited to a specific form or to a particular species of *Mucorales*. Spores which initially infect respira-

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**TABLE 3. Effect of anti-C3 antibody on serum chemotaxigenesis by Rhizopus sp. and zymosan**

<table>
<thead>
<tr>
<th>Agent preincubated with serum</th>
<th>Chemotactic activity (Cor cpm × 10^-3 ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without anti-C3</td>
</tr>
<tr>
<td>Medium 199 (control)</td>
<td>1.90 ± 0.04</td>
</tr>
<tr>
<td>Zymosan</td>
<td>2.41 ± 0.05a</td>
</tr>
<tr>
<td><em>R. rhizopodiformis</em> hyphae</td>
<td>2.25 ± 0.03a</td>
</tr>
</tbody>
</table>

*a P < 0.001.

The nature of the serum leukotactic activity was investigated by determining the effect of EDTA, EGTA, anti-C3 antibody, and prior heating on the *Mucorales* serum chemotaxigenesis (Tables 2 and 3). The pretreatment of serum by heating at 56°C or by the chelation of magnesium with EDTA prevents the activation of both the alternative and classical complement pathways. In contrast, the chelation of calcium with EGTA selectively inhibits the classical pathway, which is calcium dependent. Our studies demonstrated the generation of leukotactic activity by *Mucorales* in serum in the presence of EGTA but not in the presence of EDTA or after heating at 56°C, a pattern consistent with the activation of the alternative complement pathway. Chemotaxigenesis also failed to occur in the presence of anti-C3 or anti-factor B antibody, suggesting a lack of activity due to the unavailability of early complement components. Others have demonstrated chemotactic activity induced by *Cryptococcus*, *Coccidioides*, and *Candida* spp. through the activation of the alternative complement
pathway (9, 12, 15). In mucormycotic lesions where hyphae rapidly penetrate tissue and blood vessels, such activation of complement may provide the mechanism for attracting neutrophils and monocytes to the site of the fungal invasion of the host.

ACKNOWLEDGMENTS

This work was supported by the American Lung Association of North Carolina and the North Carolina United Way. We thank P. B. Campbell, D. Sinar, and E. Furth for critical review and P. Johnson, D. Paramore, and D. Nichols for the preparation of the manuscript.

LITERATURE CITED