Chemotaxigenic Activity of Extracts from the Mycelial and Spherule Phases of *Coccidioides immitis* for Human Polymorphonuclear Leukocytes

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Received for publication 27 June 1978

In order to further characterize human host defenses against *Coccidioides immitis*, extracts of this fungus were tested in vitro for their ability to attract polymorphonuclear leukocytes derived from peripheral blood of uninfected subjects. Soluble substances prepared from the mycelial (saprophytic) and spherule (tissue) phases exhibited, in the presence of serum, dose-dependent chemotactic activity. The dose-response correlations were different. The spherule-derived preparation showed decreased activity at the high concentrations, a diminution not observed with equivalent concentrations of the mycelial filtrate. Chemotactic activity was not observed with either substance in the absence of serum or if heat-inactivated serum was substituted. Because the response of human polymorphonuclear leukocytes to these fungal substances appears complement-mediated, a selective cellular defect in this function which antedates exposure to *C. immitis* seems unlikely.

Coccidioidomycosis is a fungal infection affecting persons who live in or travel through the endemic areas of the world. Why a small fraction of those persons exposed should develop progressive disease remains unanswered, but it appears to be due to a defect in host defenses. Previous investigations have suggested that cellular rather than humoral responses are impaired (13, 17). Such a defect would be expected to be selective since persons with more serious forms of coccidioidomycosis apparently exhibit no predisposition to other types of infections or diseases. When defects in cellular immunity are demonstrated in such patients, they are frequently specific to *Coccidioides immitis* antigens and the patients have normal responses to other antigens (5, 14).

One component of cellular defense mechanisms is the ability to mobilize leukocytes to the site of infection. A variety of substances derived from bacteria have been shown to cause migration of polymorphonuclear leukocytes (PMNs) in vitro (6). Such activity in *C. immitis* extracts has not previously been studied. Although leukotactic activity usually is mediated by the generation of complement-split fragments, some substances have displayed direct leukotactic properties in the absence of serum (6, 16). Were a similar direct leukotactic property to be found in substances derived from *C. immitis*, this might be a site for a selective cellular defect in host response to this fungus. This possibility prompted the studies to be described.

*C. immitis* is a dimorphic fungus. Elements of the saprophytic phase initially impact in the airways of the host, then undergo morphogenesis to the parasitic phase which remains predominant in the host. Antigenic dissimilarities between the two phases have been demonstrated (8, 11). For this reason we chose to study substances from both forms.

**MATERIALS AND METHODS**

Leukocytes and serum were obtained on the day of study from healthy persons who met all of the following criteria: (i) no history of coccidioidomycosis; (ii) negative skin tests to coccidioidin (1:10) and spherulin (high test strength, Berkeley Biologicals, Inc., Berkeley, Calif.); and (iii) no detectable complement-fixing antibody to *C. immitis* (performed by D. Pappagianis, University of California at Davis). Cell separation from heparinized venous blood (5 U/ml) was performed by gravity sedimentation with dextran. The leukocyte-rich supernatant (65–75% PMNs) was washed three times with Dulbecco buffered saline. The cell pellet was then resuspended in medium-199 supplemented with 2% bovine serum albumin to a final concentration of 5 × 10⁶ PMNs/ml.

Two fungal-derived substances were studied. Mycelial filtrate, the filtered medium that had supported the growth of the saprophytic phase of *C. immitis*, was prepared and kindly supplied by D. Pappagianis. This

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material was dialyzed twice against normal saline before use, yielding a retentate whose concentration was 4.9 mg/ml (4). Spherulin, the soluble fraction of a hypotonic lysate of washed spherules (10), was generously supplied by Berkeley Biologicals, Inc., at a concentration of 1.83 mg/ml. Most of our studies with spherulin were performed without a dialysis step. Although parallel comparison of dialyzed and undialyzed preparations was not performed, four consecutive studies with the two preparations yielded comparable results. To exclude the possibility of endotoxin contamination of our materials, mycelial filtrate and spherulin were shown to be without activity as determined in the in vivo rabbit assay for the pyrogenic property of endotoxin (these studies were kindly performed by Phyllis Bodel, Yale University).

For studies where dialyzed mycelial filtrate or spherulin was studied in the presence of serum, various saline dilutions of the fungal substance were combined with equal parts of serum (derived from the same donor as the cells) and incubated first at 37°C for 10 min and then at 56°C for 30 min. The mixture was then diluted with 1.5 volumes of saline for use.

For studies where serum was absent, the fungal substances were prepared as saline dilutions without the incubation steps described above. A staphylococcal filtrate was prepared by filtration of the supernatant of an overnight growth of S. aureus 502A in brain heart infusion broth through a 0.45-μm filter. This served as a positive control (6).

Chemotaxis studies were performed in stainless-steel Boyden chambers (Belloco, Vineland, N.J.). These consist of upper and lower compartments, separated by a 25-mm ester of polycarbonate filter (Millipore Corp., Bedford, Mass.) which has an average pore size of 3 μm. These filters do not admit mononuclear cells (7). The attracting mixture to be studied was injected into the bottom compartment, and 1 ml of the leukocyte suspension was pipetted into the upper compartment. Chambers were then placed at 37°C for 90 min. After this incubation, the filters were removed, fixed with ethanol, stained with hematoxylin, cleared with xylene, and mounted on microscope slides.

Migration was quantitated by counting PMNs at a depth within the filter standardized by the activity of the autologous serum controls for each day’s experiment. Starting the examination at the lower surface of the filter and focussing upward with the microscope, the depth was found at which serum caused migration of 5 to 15 PMNs/high-power field (the average of eight fields counted per filter and two or more replicate filters per experiment). All other filters were then counted in a similar manner at that depth.

RESULTS

Response to dMF and spherulin in the presence of serum. Dialyzed mycelial filtrate (dMF) and spherulin both exhibited chemotactic activity in the presence of serum. This activity was found to be dependent on the concentration of the fungal-derived substance as depicted in Fig. 1 and 2. With dMF, a plateau was reached at approximately 200 μg/ml. In contrast, there was a marked decrease in activity at the highest spherulin concentrations tested which was not observed at equivalent concentrations of dMF. The chemotactic activity was lost if serum incubated at 56°C for 30 min was substituted for unheated serum. Tables 1 and 2 show representative data demonstrating this loss for dMF and spherulin, respectively.

Response to dMF and spherulin in the absence of serum; controls. The lack of activity in the presence of heat-inactivated serum suggested that neither dMF nor spherulin had direct chemotactic activity for PMNs. This in fact was found when these two substances were used in the absence of serum as shown in Table 3. Because these data represent counts at a filter depth where the serum controls give a standardized response, it is possible that this method of quantitation might not detect a lower level of response as compared against saline alone. However, microscopic review of the filters uniformly demonstrated that cells migrated less than 5 μm in response to either dMF or spherulin, which was identical to the findings with saline alone. A dialysate of the uninoculated medium used to
Table 1. Chemotaxis toward mycelial-phase substances with unheated or heat-inactivated autologous serum

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Chemotactic response (PMN/HPF ± SE)*</th>
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<tbody>
<tr>
<td>20% unheated serum</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>20% unheated serum + dialyzed mycelial filtrate (50 µg/ml)</td>
<td>81 ± 16</td>
</tr>
<tr>
<td>20% heat-inactivated serum + dialyzed mycelial filtrate (50 µg/ml)</td>
<td>7 ± 4</td>
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</tbody>
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*Polymorphonuclear leukocytes/high-power field ± standard error.

Table 2. Chemotaxis toward spherule-phase substances with unheated or heat-inactivated autologous serum

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Chemotactic response (PMN/HPF ± SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% unheated serum</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>20% heat-inactivated serum</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>20% unheated serum + spherulin (123 µg/ml)</td>
<td>46 ± 8</td>
</tr>
<tr>
<td>20% heat-inactivated serum + spherulin (123 µg/ml)</td>
<td>4 ± 1</td>
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*Polymorphonuclear leukocytes/high-power field ± standard error.

Table 3. Chemotaxis to cocciidioidal substances in the absence of serum

<table>
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<tr>
<th>Stimulus</th>
<th>Chemotactic response (PMN/HPF ± SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline alone</td>
<td>0</td>
</tr>
<tr>
<td>Unheated autologous serum</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Staphylococcal filtrate (20%)</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>Dialyzed mycelial filtrate (392 µg/ml)</td>
<td>0</td>
</tr>
<tr>
<td>Spherulin (123 µg/ml)</td>
<td>0</td>
</tr>
</tbody>
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*Polymorphonuclear leukocytes/high-power field ± standard error.

Heat-inactivated serum is substituted, this activity is lost. Our observations suggest that the PMN migration is mediated by heat-labile serum factors, probably those of complement. Thus, it would be useful to investigate directly the interaction of these substances and the complement system. Furthermore, our work fails to demonstrate a direct chemotactic response of PMNs to these substances. Thus, if defects in PMN chemotaxis exist which antedate and predispose patients to progressive coccidioidomycosis, these are not likely to be cellular defects specific for C. immitis. Cellular defects, humoral deficiencies, or inhibiting substances associated with progressive disease are not excluded by this study. However, the study of the specificity of such perturbations would likely be complicated by the nonspecific influences of active disease.

The diminished PMN leukotactic activity at very high concentrations of spherulin could have significance in host defenses. Histologically, the inflammatory response is characterized by PMNs at the primary site of infection by the saprophyte phase of C. immitis. After conversion of the fungus to the parasitic phase, PMNs are found at the time of spherule rupture and initiation of fungal multiplication (1). Although PMN function in these lesions remains obscure, in vitro studies demonstrate that they are capable of phagocytosis of endospores (S. C. Deresinski, H. B. Levine, and D. A. Stevens, Mycopathology, in press) and that PMNs affect the rate of conversion of arthropores to spherules (1). Killing of C. immitis by PMNs has not been demonstrated as has been done with candida (9). However, if any interaction of PMNs with C. immitis is important in the host defense, then the inhibition of migration of PMNs by high concentrations of spherule-derived substances might obstruct efforts to limit the infection.

Mononuclear cells are found in sites of C. immitis infection, particularly in association with growing parasitic-phase structures (15). Since they could play a role in limiting infection by phagocytosis, granuloma formation, or production of factors inducing fibroblast proliferation, it will be of interest to similarly examine whether cocciidioidal substances exert specific, noncomplement-mediated attraction for these cells.

Discussion

Mycelial filtrate and spherulin have concentration-dependent chemotactic activity for human PMNs in the presence of serum. These findings are similar to those with particulate and filterable substances from species of candida (3, 12), although others have reported preparations with serum-independent chemotactic activity (2). In addition, when the serum is deleted or

acknowledgments

Supported by grants from The Medical Research and Education Fund, California Lung Association, and the John A. Hartford Foundation.

Literature Cited

C. IMMITIS LEUKOCYTE CHEMOTAXIS