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Interactions Between Human Granulocytes and *Blastomyces dermatitidis*

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We studied interactions in vitro between human granulocytes and the yeastlike form of *Blastomyces dermatitidis*, because granulocytes are prominent in the host response to systemic blastomycosis. In Boyden chamber assays, broth culture filtrates of *B. dermatitidis* contained levels of granulocyte chemotactic activity that were significantly higher than those present in similar culture filtrates of *Histoplasma capsulatum* and *Cryptococcus neoformans*, two fungi that characteristically do not elicit granulocytes in infected tissues. Microscopic study, including electron microscopy, demonstrated that granulocytes phagocytosed *B. dermatitidis* promptly and efficiently. Moreover, granulocytes emitted light (chemiluminescence) at a brisk rate during phagocytosis of *B. dermatitidis*, indicating activation of intracellular metabolic pathways. However, fungicidal assays showed that granulocytes (1:1 cell-yeast ratio, 10% serum) killed only 29% of the *B. dermatitidis* inoculum during 3 h of incubation. Taken together, these findings suggest that there is disparity between phagocytosis and intracellular killing of *B. dermatitidis* by human granulocytes, perhaps because of resistance of this fungus to granulocyte microbicidal mechanisms.

The host response to *Blastomyces dermatitidis*, the dimorphic fungus that causes systemic blastomycosis, is characteristically suppurative as well as granulomatous; infected tissues are usually infiltrated by granulocytes (4, 8). Despite this consistent observation, there is only limited information (21) on the capability of the human granulocyte to contribute to host defense against this fungus. The present study was designed to examine interactions in vitro between human granulocytes and the yeastlike phase of *B. dermatitidis*, which is the form of the fungus found in human tissues. Specific granulocyte functions that were studied represent sequential steps in the process by which granulocytes locate and kill invading microbes: chemotaxis, phagocytosis, metabolic activation, and intracellular killing (19).

MATERIALS AND METHODS

Organisms. One strain each of *B. dermatitidis*, *Cryptococcus neoformans*, and *Histoplasma capsulatum* were selected from a stock of clinical isolates from patients with systemic mycotic infections. They were maintained on Sabouraud agar at 37°C with biweekly transfer.

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Chemotaxis assays. Pine broth (15) was inoculated with a loopful of stock culture of each of the three test fungi or, as a control, was processed without fungal inoculation. Inoculated broth was incubated at 37°C for 48 h, filtered, and stored at −70°C. Filtrates were tested for chemotactic activity by a previously described assay (9) employing ^{51}Cr-labeled human granulocytes and a two-filter Boyden chamber system. The lower compartments of the chambers contained fungal broth culture filtrates in concentrations (vol/vol) of 1, 2.5, and 10% in Gey medium (Microbiological Associates, Bethesda, Md.). Chambers were incubated for 3 h at 37°C, and chemotaxis was quantitated by determining the counts per minute of ^{51}Cr in the lower filters from each of four replicate chambers.

Chemiluminescence. Photon emission from incubating mixtures of granulocytes and *B. dermatitidis* was measured in a liquid scintillation counter (Beckman liquid scintillation system L-200) by a previously described method (18). To dark-adapted scintillation vials were added, in sequence, 10^7 granulocytes and 10^7 *B. dermatitidis* in 5 ml of Hanks balanced salt solution (Baltimore Biological Laboratory Division, Becton, Dickinson & Co., Cockeysville, Md.) supplemented with gelatin, 1 mg/ml, pH 7.4 (Hanks-gel), with 10% serum. Fungi were withheld until a background count was obtained for 10 min. After thorough mixing, counts were obtained (out-of-coincidence mode, tritium window, ambient temperature) every minute for 45 min without further agitation of the tubes. In some experiments fungi were replaced by

Staphylococcus aureus at a granulocyte:bacteria ratio of 1:10, or by zymosan A (Sigma Chemical Co., St. Louis, Mo.), 1 mg/ml.

Phagocytosis and fungicidal activity. *B. dermatitidis* was cultured in Pine broth for 64 h at 37°C, washed three times and suspended in calcium and magnesium-free Hanks balanced salt solution, pH 7.4 (Baltimore Biological Laboratory). Aggregates that were characteristically formed during growth in broth were dispersed by sonification for 30 s at 4°C. Fungi were counted in a hemocytometer, and the organisms were suspended in Hanks-gel at a concentration of 10^7 organisms per ml. Granulocytes from peripheral blood of healthy male volunteers were prepared by dextran sedimentation as previously described (9) and suspended in Hanks-gel at 10^7 granulocytes per ml. Such preparations were comprised of 70 to 90% granulocytes with average viability greater than 95% by trypan blue exclusion. Serum of AB blood type was obtained from a 24-year-old male volunteer who had a negative *B. dermatitidis* skin test and lacked complement-fixing serum antibody against *B. dermatitidis*. Convalescent serum was donated by a patient treated 1 year previously for pulmonary blastomycosis. His disease was inactive, he had negative sputum cultures for *B. dermatitidis*, and he was also seronegative.

Granulocyte fungicidal assays were performed by adding to tubes (12 by 75 mm) 2×10^6 granulocytes, 10% serum, 2×10^6 or 2×10^5 *B. dermatitidis* (granulocyte to fungus ratios of 1:1 or 10:1, respectively), and Hanks-gel, to bring the total volume to 1 ml. Control tubes containing all components except granulocytes were included in each experiment, and all specimens were run in duplicate. Immediately after mixing the components and at subsequent intervals of 1, 2, and 3 h, aliquots were removed from each tube for microscopic examination and determination of viable organisms by plating serial dilutions on Sabouraud agar and performing colony counts after culturing at room temperature for 10 to 14 days.

Electron microscopy. Electron microscopy was performed on preparations of *B. dermatitidis*, serum, and granulocytes after incubation for 30 min and 2 h. Samples were processed and stained as previously described (20). Electron micrographs were taken on a Siemens 101 electron microscope.

RESULTS

Chemotaxis studies. The broth culture supernatant of the experimental strain *B. dermatitidis* contained granulocyte chemotactic activity that was significantly greater than that demonstrated in supernatants of *C. neoformans* and *H. capsulatum* (Table 1).

Chemiluminescence. Granulocytes interacting with *B. dermatitidis* emitted light at a brisk rate (Fig. 1). Granulocyte chemiluminescence stimulated by *B. dermititidis* was less than that invoked by zymosan, but more than that caused by *S. aureus* under the conditions employed. An additional study using granulocytes from another donor gave similar results (peak, 2.76 × 10^4 cpm above base line after 11 min of incubation).

Microscopic assessment of phagocytosis. Examination of granulocyte-serum-yeast mixtures by light microscopy demonstrated that

TABLE 1. *Granulocyte chemotactic activity in supernatants of fungal broth cultures*

Supernatant concn[a]	Chemotaxis (cpm)[b]			
	Pine broth control (5)[c]	*B. dermatitidis* (5)	*C. neoformans* (3)	*H. capsulatum* (3)
1.0	278 ± 54[d]	754 ± 95 (<0.02)[e]	349 ± 28	287 ± 64
2.5	191 ± 27	1,039 ± 122 (<0.025)	409 ± 80	485 ± 122
10.0	290 ± 69	1,633 ± 329 (NS)	677 ± 160	969 ± 190

[a] Percentage by volume of broth culture supernatant in lower compartment of Boyden chambers. Buffer was Gey medium, pH 7.4.

[b] Counts per minute from lower of two filters separating ^{51}Cr-labeled granulocytes and fungal broth culture supernatants after 3 h of incubation at 37°C in Boyden chambers.

[c] Number of experiments.

[d] Mean ± standard error.

[e] Level of significance of difference (*P*) from mean counts per minute of other fungal culture supernatants. NS, Not significant.

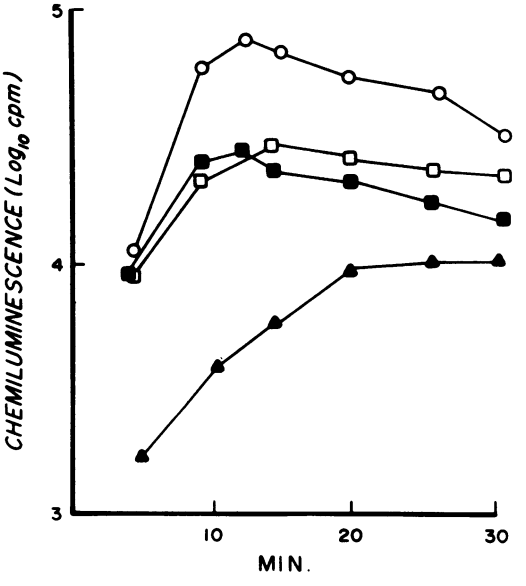

FIG. 1. *Granulocyte chemiluminescence during phagocytosis of: B. dermatitidis (1:1 granulocyte:yeast ratio), 10% control serum (■); 10% convalescent serum (□); zymosan, 1 mg/ml (○); and S. aureus (1:10 granulocyte:bacteria ratio), 10% control serum (▲). Measured on the ordinate was proton emission from dark-adapted phagocytic preparations. The granulocyte concentration in all preparations was 10^7/ml.*

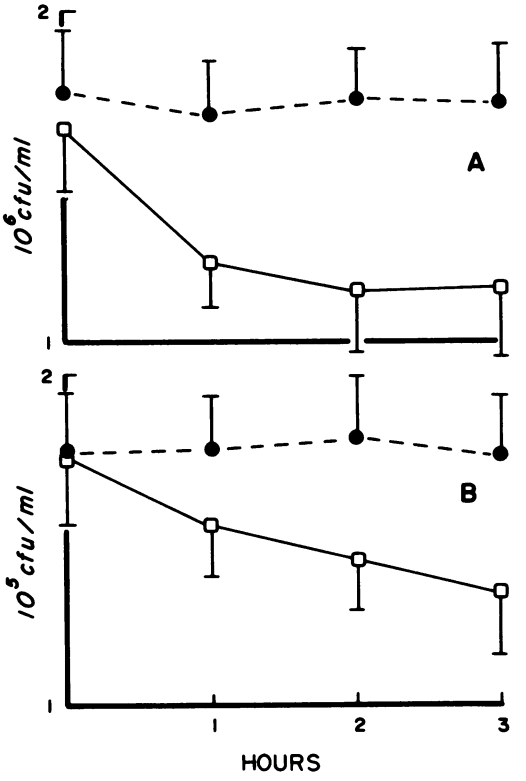

FIG. 2. *Reduction of B. dermatitidis CFU during incubation with granulocytes in Hanks-gel buffer and 10% control serum. Granulocyte:yeast ratios were 1:1 (A) and 10:1 (B) in preparations with means (± standard error) marked (□); preparations with means marked (●) contained yeasts and control serum, but no granulocytes. Level of significance of difference (P) between both sets of paired means was <0.05 at 2 and 3 h.*

after 30 min of incubation, more than 95% of *B. dermatitidis* cells were associated with granulocytes. Most aggregates were composed of 10 to 50 fungal particles and an equal number of granulocytes; it was difficult to determine whether fungi were intracellular. Control smears of granulocytes alone and *B. dermatitidis* alone in the buffer-serum solution showed virtually no aggregation.

Electron microscopy gave further proof of *B. dermatitidis* phagocytosis by granulocytes. In most instances granulocytes ingested a single fungal cell, but there were also examples of phagocytosis of two or three *B. dermatitidis* by one granulocyte, as well as those of interaction of one fungus with two or more granulocytes. After 30 min of incubation, 145 or 147 consecutive fungal cells (99%) were located within granulocytes, and 19 (13%) of the cells were morphologically abnormal as judged by disintegration of cell organelles. After 2 h of incubation, 39 (15%) of 263 intracellular and 14 (24%) of 59 extracellular fungi showed similar alterations. When *B. dermatitidis* were incubated in serum without granulocytes, 19 (6%) of 300 fungi were morphologically abnormal.

Fungicidal studies. Exposure of *B. dermatitidis* to granulocytes (1:1 ratio) and 10% serum for 3 h resulted in a mean reduction in colony-forming units of 29% (Fig. 2). The results of preparations containing one fungus to 10 granulocytes were similar. Serum concentrations higher than 10% resulted in no significant augmentation of killing (20 and 40% serum gave 31 and 36% reduction in colony-forming units [CFU], respectively); however, in the absence of serum there was no reduction in CFU. There was no enhancement of granulocyte fungicidal activity when normal serum was replaced by convalescent serum (19% CFU reduction at 1:1 granulocyte:yeast ratio, mean of five experiments).

DISCUSSION

These experiments show that human granulocytes respond to *B. dermatitidis* as they do to pyogenic bacteria. First, granulocytes were highly stimulated by chemotactic factors elaborated by *B. dermatitidis* during growth in broth culture. Several types of bacteria and *C. albicans* produced granulocyte chemotactic factors during growth (5, 20, 22), but this property has not previously been associated with growth of *B. dermatitidis*. The chemotactic activity elaborated by *B. dermatitidis* was greater than that produced by growing cultures of *H. capsulatum* and *C. neoformans*, two fungi that characteristically do not elicit a granulocyte response. However, these comparisons should be interpreted with caution since there was no attempt to standardize the cultures of different fungi in terms of concentrations of organisms, protein, or polysaccharide in the filtrates.

Secondly, phagocytosis of *B. dermatitidis* by granulocytes was rapid and efficient and was associated with activation of granulocyte oxidative metabolism as marked by chemiluminescence. Pyogenic bacteria also stimulate strong chemiluminescence as they are phagocytosed by granulocytes (14, 17, 18). Granulocyte chemiluminescence during phagocytosis may relate to the formation of microbicidal substances such as singlet oxygen or superoxide anion (2), or to the myeloperoxidase-hydrogen peroxide-halide microbicidal system (16). Chemiluminescence may also be stimulated by events other than phagocytosis, such as exposure of the cells to soluble chemoattractants (1).

Despite this normal sequence of responses, the capability of human granulocytes to kill intracellular *B. dermatitidis* is modest, compared to their ability to kill bacteria. When bacteria are ingested by normal human granulocytes, they are rapidly killed (19), but *B. dermatitidis* survives successfully within human granulocytes according to the present data (Fig. 1) that showed only 29% killing of the initial fungal inoculum in spite of phagocytosis of greater than 95% of the organisms. The occurrence of aggregates of granulocytes and fungi in microscopic smears actually raises the possibility that the fungicidal assay underestimated the number of viable fungi. Thus, our data should be regarded as determining a maximum value for granulocyte fungicidal activity under the conditions employed.

It is not surprising that the susceptibility of fungi to granulocyte microbial mechanisms is different than that of bacteria. For example, their cell walls are distinctly different with respect to ultrastructure, biochemical content, and resistance to changes in pH (13). Other investigators are now studying novel mechanisms by which granulocytes act against fungi, including extracellular killing (3, 6, 7, 11); these techniques need to be applied to the study of *B. dermatitidis*.

The clinical implications of this study deserve comment. On the one hand, the abundance of granulocytes in tissues invaded by *B. dermatitidis* (4, 8) could be related to the production of chemotactic factors by the fungus. However, in view of the apparent ease with which granulocytes phagocytose *B. dermatitidis*, it is somewhat surprising that the yeastlike forms are rarely found within granulocytes in tissue sections. Finally, it is tempting to speculate that the relative resistance of *B. dermatitidis* to intracellular killing by human granulocytes is somehow related to the relentless progression of untreated systemic blastomycosis (12).

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