INFECTION AND IMMUNITY, Oct. 1984, p. 87–93
0019-9567/84/100087-07$02.00/0

Vol. 46, No. 1

Blastomyces dermatitidis Chemotactic Factor: Kinetics of Production and Biological Characterization Evaluated by a Modified Neutrophil Chemotaxis Assay

LINDA M. THURMOND AND THOMAS G. MITCHELL*

Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710

Received 8 May 1984/Accepted 6 July 1984

Chemotactic activity for human polymorphonuclear neutrophils (PMNs) was detectable in culture filtrates (CFs) of *Blastomyces dermatitidis* and may have influenced the pathogenesis of blastomycosis. Production of this chemotaxin depended upon culture age and medium; peak levels were achieved after incubation for 17 days or more in minimal essential medium. This factor was also chemotactic for human monocytes. CF was temperature stable even after treatment at 100°C for 60 min. The activity was stable under alkaline conditions but was destroyed below pH 7. Dialyzed, chemotactically active CF contained approximately 60 μg of carbohydrate per ml; total protein was estimated to be less than 0.8 μg/ml. Preincubation of PMNs with CF or *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine deactivated their chemotactic response to each agent, whereas the chemotactic response to zymosan-activated serum was not affected. In addition, deactivation with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine reduced the response to CF. Pretreatment of CF with PMNs decreased chemotactic activity, which may reflect binding of the chemotaxin molecules to PMN receptors. A modified chemotaxis assay was developed in which commercial, disposable multiwell plates are used. This method was rapid, efficient, and inexpensive and permitted the assay of larger numbers of samples than was previously feasible with conventional chemotaxis methods.

Blastomycosis causes a wide range of clinical manifestations. Pulmonary lesions may be cavitary, granulomatous, or ulcerative. In the skin, yeast cells of *Blastomyces dermatitidis* may be found in dermal microabscesses at the advancing border of the lesions. Both suppurative and granulomatous tissue responses characterize blastomycotic lesions. Polymorphonuclear neutrophils (PMNs) initially accumulate around the yeast cells, and macrophages and fibroblasts subsequently migrate to the periphery of the microabscesses (2, 11).

Interactions between *B. dermatitidis* and PMNs have been investigated in several laboratories. The sigificance of PMNs in blastomycosis is suggested by the report of an inhibitor of PMN chemotaxis in the sera of patients with untreated systemic blastomycosis but not other mycoses (16). However, the replication of *B. dermatitidis* after subcutaneous injection into mice is enhanced by the presence of PMNs (6). Indeed, intact PMNs or lysates stimulate the replication of *B. dermatitidis* in vitro (5), although the susceptibility of *B. dermatitidis* to products of oxidative metabolism has also been demonstrated (18).

The first report of a fungal chemotactic factor demonstrated that serum-free culture filtrates of *Candida albicans* attract guinea pig neutrophils (7). Chemotactic activity has since been reported in 48-h culture filtrates of *B. dermatitidis* in Pine medium (17). The kinetics and culture medium requirements for production of the *B. dermatitidis* chemotactic factor were subsequently investigated in this laboratory (L. M. Thurmond, T. G. Mitchell, and S. R. Turner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, F1, p. 326). Peak chemotactic activity was observed between 18 and 38 days. Production of this factor was greater when the yeasts were grown in tissue culture medium 199 (TC199) at 37°C, lower in brain heart infusion broth, and least in glucose-yeast extract broth (12); chemotactic activity did not correlate with the amount of growth in each medium. The chemotactic factors derived from culture filtrates of both *C. albicans* and *B. dermatitidis* are resistant to 90°C, freezing, and prolonged storage, and both are retained by filters having an estimated retention limit of 10,000 daltons (7, 12).

Although the yeast cells of *B. dermatitidis* (like those of other fungi) generate complement-mediated chemotaxins (unpublished observations), production of a serum-independent chemotaxin may explain the presence of high numbers of PMNs (and macrophages) in blastomycotic lesions. As a first step toward investigating this hypothesis, the chemotactic factor was produced in vitro under defined conditions and its biological properties were evaluated.

MATERIALS AND METHODS

Cultures and filtrates of *B. dermatitidis*. Stock cultures of *B. dermatitidis* derived from ATCC 26199 (gifts from Rebecca A. Cox, San Antonio State Chest Hospital, San Antonio, Tex., and Elmer Brummer, Santa Clara Valley Medical Center, San Jose, Calif.) were stored on slants of medium containing 2% (wt/vol) glucose, 1% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.), and 2% (wt/vol) agar at −20°C. Cultures were thawed, and 25-ml shaker cultures were established in the following test media: TC199 (Microbiological Associates, Walkersville, Md.), minimal essential medium with L-glutamine and $NaHCO_3$ (MEM), diluted from 10× stock (Flow Laboratories, Inc., McLean, Va.) with pyrogen-free water, or carbon base medium (CBM) (Difco) supplemented with 1.5 g of asparagine and 3.5 g of ammonium sulfate per liter and dissolved in 0.01 M potassium phosphate buffer at either pH 7.5 or 6.0 (CBM-PB). Primer cultures in each medium were incubated for 4 days at 37°C in a rotary shaker (model G-25; New Brunswick Scientific Co., Inc., Edison, N.J.) at 150 rpm and used to inoculate flasks of homologous media at a density of 10^5 yeast cells per ml as determined by hemacytometer count. For sampling of chemotactic activity, portions of culture

* Corresponding author.

fluid were removed aseptically, filtered through a membrane filter (0.45-μm porosity; Millipore Corp., Bedford, Mass.), and stored at −20°C. For quantification of growth, samples of culture fluid were filtered through preweighed filters and dry weights were determined. The pH of each culture medium was determined from a filtered 1-ml sample. After incubation for 45 days, cultures were harvested by centrifugation and filtration and stored at −20°C. The sterile fluid was concentrated by rotary evaporation (Buchi Rotavapor; Brinkmann Instruments, Inc., Westbury, N.Y.), dialyzed against 0.1 M Tris-buffered saline, pH 7.4 (Spectra-Por dialysis tubing with a nominal molecular weight cutoff of 3,500; Spectrum Medical Industries, Inc., Los Angeles, Calif.), and stored at 4°C; this fluid is referred to here as culture filtrate (CF).

Glassware used for all procedures was depyrogenated before use by dry heat; pyrogen-free disposable labware was utilized whenever possible. Heat-sensitive, recycled equipment (e.g., blind-well chambers) was cleaned after each use (Micro Cleaner; International Products Corp., Trenton, N.J.), rinsed with pyrogen-free water, and promptly dried under a heat lamp. The *Limulus* amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, Mass.), used to test reagents and media for the presence of endotoxin, has a reported sensitivity of 10 ng of *E. coli* endotoxin standard per ml. The purity of cell cultures and the sterility of culture filtrates were confirmed by standard microbiological methods at every step of the procedures described.

Neutrophils and monocytes. Leukocytes for chemotaxis assays were freshly isolated from whole human peripheral blood obtained from healthy volunteers. Cells were allowed to sediment by gravity through 3% dextran (Sigma Chemical Co., St. Louis, Mo.) in 0.85% NaCl, and the supernatant was centrifuged through lymphocyte separation medium (Litton Bionetics, Inc., Kensington, Md.). Monocytes were removed from the interface and transferred to sterile Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) (HBSS) supplemented with 0.5% (wt/vol) bovine serum albumin (Sigma) (BSA), pH 7.4 in the pellet. Erythrocytes were lysed with 0.1% NaCl, and the suspension was restored to isotonicity with 1.7% NaCl before centrifugation and resuspension of neutrophils in HBSS-BSA. Differential counts were done in a hemacytometer by counting cells stained with crystal violet. Cells were suspended in HBSS-BSA at the appropriate density, and viability was determined by exclusion of 0.04% trypan blue (GIBCO).

Preparation of chemoattractants. Zymosan-activated serum (ZAS) was prepared from fresh human serum by incubating 25 mg of saline-washed zymosan A (Sigma) with 1 ml of serum for 30 min at 37°C. After incubation, the zymosan was removed by centrifugation and the supernatant was stored undiluted at −20°C.

The synthetic peptides *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (f-MLF) and *N*-formyl-L-methionyl-L-phenylalanine (Sigma) were dissolved in methanol at a concentration of 1 mg/ml and stored at −20°C. For assay, a sample of stock solution was dried under nitrogen, resuspended in HBSS-BSA by vigorous vortexing, and diluted appropriately.

Bacterial factor was prepared by growing *Escherichia coli* JA199/DU650 (generously furnished by the late R. O. Burns, Duke University Medical Center, Durham, N.C.) in MEM for 24 h at 37°C with shaking. After incubation, the cultures were centrifuged, and supernatant fluids were decanted and filtered through membrane filters (0.45-μm porosity) and stored at −20°C in small volumes.

Chemotaxis assays. Chemotaxis was initially evaluated as previously described (12, 19), using blind-well chambers fitted with nitrocellulose filters having porosities of 3 or 8 μm (Sartorius Filters, Inc., Hayward, Calif.) for examination of neutrophil or monocyte responses, respectively. Each chemotactic agent was placed in the lower well and separated from a 330-μl suspension of 2×10^6 PMNs or 5×10^6 monocytes per ml in the upper reservoir by a filter (13 mm in diameter). Positive chemotaxis controls included 2 μM *N*-formyl-L-methionyl-L-phenylalanine, 20 nM f-MLF, 1% (vol/vol) ZAS, and 10% (vol/vol) bacterial factor. The diluent for these chemotaxins, HBSS-BSA, was the negative control. The assembled blind-well chambers were incubated for 35 and 85 min for assay of PMNs and monocytes, respectively, in a humidified incubator at 37°C with 5% CO_2. The filters with cells were stained with hematoxylin and mounted on microscope slides as previously described (19). Chemotaxis was quantitated by the leading-front method (21) with a modified, semiautomated data analysis system (19). All controls and putative chemoattractants were assayed in triplicate, with five measurements of migration distance taken per filter; each experiment was performed at least twice. Chemotaxis data are expressed as the mean distance in micrometers (± standard deviation) migrated in three filters by the leading two PMNs in five high-power fields per filter (19, 21).

The primary method of assaying chemotaxis in this laboratory was developed as a modification of other multiwell chamber methods (1, 10, 14). In this method, disposable multiwell plates (catalog no. 010102201; Dynatech Laboratories, Inc., Alexandria, Va.) were employed. A thin, even layer of silicone vacuum grease was applied to the upper surface of the plate, and alternate wells were filled with 320 μl of chemotactic agents. Sartorius filters (13 mm in diameter) were positioned over the wells with vacuum forceps and allowed to drop onto the grease layer. Neutrophils suspended in HBSS-BSA at 2×10^7 PMNs per ml were placed onto each filter in 10-μl drops. The plates were incubated in a humidified incubator at 37°C with 5% CO_2, after which time the filters were fixed, stained, mounted, and evaluated as described above.

Statistical analysis of chemotaxis data was performed by the Student *t* test, and derived *P* values are indicated for all appropriate experiments. The coefficient of variation (between runs) was calculated for both chemotaxis methods.

Checkerboard assays were performed as described by Zigmond and Hirsch (21). CF was tested at 1.25, 2.5, 5, and 10% (vol/vol), with HBSS-BSA serving as the diluent and negative control.

Protein and carbohydrate assays. Protein concentration was determined by the method of Bradford (3), as available from Bio-Rad Laboratories, Richmond, Calif. Carbohydrate was assayed by the phenol-sulfuric acid method of Dubois et al. (9).

Temperature stability. The thermal stability of the chemotactic factor was evaluated in three ranges. A sample of CF was subjected to 100°C for 60 min and another was held at 56°C for 60 min, and both samples were then cooled to 37°C. Another sample was frozen at −20°C and then brought to 37°C for three cycles. All samples were adjusted to pH 7.4, diluted, and assayed for chemotactic activity as compared with an untreated, control CF.

pH stability. Separate samples of CF were adjusted to various pH values between 3 and 10 for 18 to 24 h at 4°C and then adjusted to pH 7.4 with either 1.0 N NaOH or 1.0 N HCl and brought to equal volumes. For the evaluation of

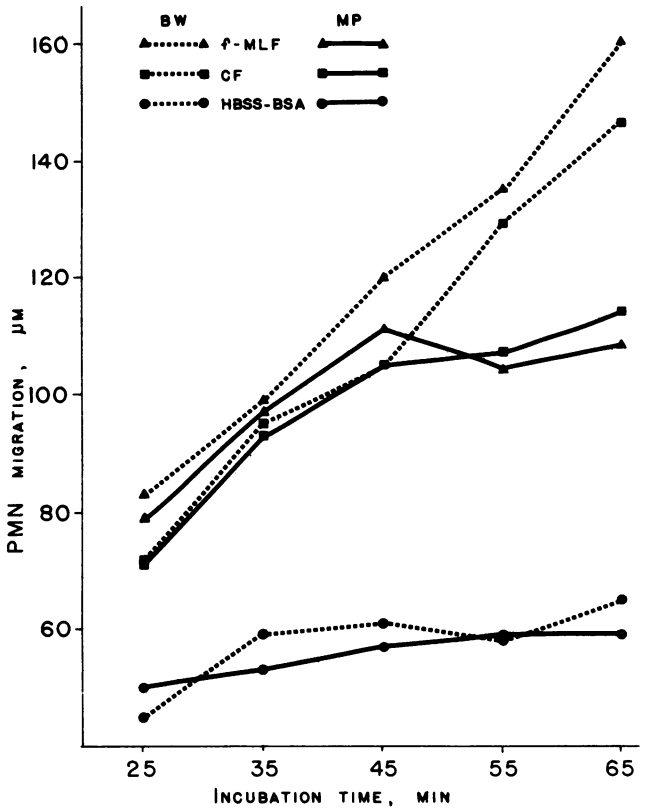

FIG. 1. Comparison of neutrophil chemotaxis through membrane filters (3-μm porosity) mounted in blind-well chambers (BW) or on multiwell plates (MP) and incubated for various times. Migration distances were quantitated by the leading-front method and evaluated for the diluent control (HBSS-BSA), 10% (vol/vol) *B. dermatitidis* CF, and 20 nM f-MLF.

chemotactic activity, each sample was diluted and assayed at final concentrations of 5, 10, and 20% (vol/vol) in HBSS-BSA; untreated, control CF was similarly diluted and tested.

Deactivation of neutrophil chemotaxis. PMNs isolated as previously described were washed and resuspended to 0.5 ml in HBSS-BSA, 2 μM f-MLF, or a ten-fold concentration of dialyzed CF (the final concentration of each agent was 100-fold greater than the optimal chemotactic dilution). Cells were incubated in the buffer control or in the test agents for 60 min at 25°C, washed three times with HBSS (without BSA), and then resuspended in fresh HBSS-BSA for chemotaxis assay. Chemotaxis of deactivated neutrophils was examined in response to HBSS-BSA (negative control for random migration and diluent) and to optimal concentrations of f-MLF, ZAS, and CF.

Adsorption of chemotactic activity in CFs by neutrophils. PMNs fixed in 10% formaldehyde were washed extensively with HBSS, and 3×10^4, 3×10^5, or 3×10^6 cells were dispensed into polypropylene tubes containing phosphate-buffered saline (pH 7.4) and then centrifuged. The cell pellet in each tube was resuspended in 300 μl of CF and incubated at 25°C. After incubation for 60 min, cells were removed by centrifugation and the supernatants were assayed for chemotactic activity at dilutions of 10 and 20% (vol/vol). In some experiments, viable PMNs were used as the adsorbant. Controls included CF incubated without PMNs.

RESULTS

Evaluation of the multiwell plate method. The migration distances of PMNs in response to chemotaxins or HBSS-BSA in both chemotaxis methods were compared to determine the optimal incubation time for the multiwell plate method. Chambers and plates were prepared concurrently with the same batch of PMNs. All the membrane filters used had the same lot number and measured 175 μm in thickness after fixation, staining, and mounting. Migration toward positive controls in the multiwell plate system increased rapidly during the first 45 min (Fig. 1). In the multiwell assay, a smaller fluid volume is employed above the filter, and beyond 45 min, the chemotactic gradient may have been diminished. The distance of random migration to HBSS-BSA did not increase appreciably after 35 min of incubation. In both assay systems, PMN migration in response to the optimal concentration of *B. dermatitidis* CF was usually less than the distance migrated toward f-MLF.

At 35 min of incubation, the multiwell plate method yielded chemotaxis values similar to those of the established blind-well chamber method. The values obtained with both methods were reproducible; the standard deviation of the mean values was ≤10%. Eight separate experiments in which we compared the two methods (with different batches of PMNs and filters) were used to calculate the between-run percent coefficients of variation for one negative and two positive controls. The mean coefficients of variation for the blind-well and multiwell chamber methods were 8.2 and 8.0%, respectively.

The correlation between the values obtained with the chamber and the multiwell plate assays at 35 min was excellent (Fig. 2); the rank correlation coefficient was 0.975 ($P < 0.001$). In the multiwell plate method, fewer cells are employed per triplicate sample, i.e., 6×10^5 PMNs compared with the 2×10^6 PMNs required for the blind-well chamber assay. Cell viability during incubation at both cell densities was ≥99%, as determined by trypan blue dye exclusion.

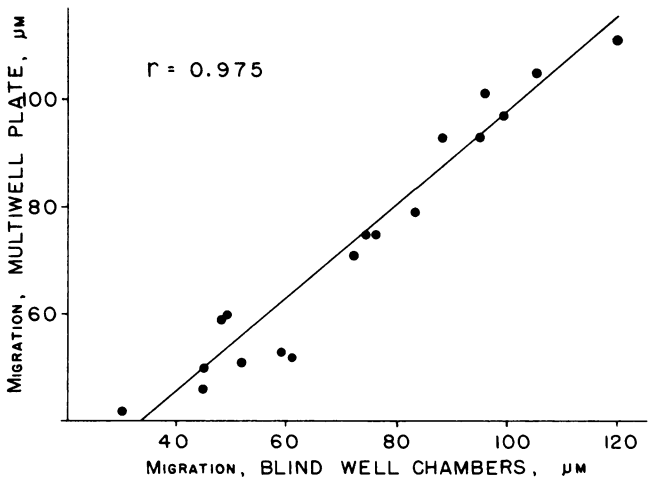

FIG. 2. Correlation of neutrophil migration distances in blind-well chambers and multiwell plates for parallel 35-min incubations. Each point represents the migration distances for chemotaxins (10% *B. dermatitidis* CF or 20 nM f-MLF) or diluent (HBSS-BSA) which were tested simultaneously in blind-well chambers or multiwell plates. Data shown are from two separate experiments. r, Correlation coefficient.

TABLE 1. Checkerboard analysis by the multiwell plate method of neutrophil chemotaxis to *B. dermatitidis* CF

% (vol/vol) CF above filter (with cells)	% CF (vol/vol) below filter[a]:				
	0	1.25	2.5	5.0	10.0
0	53	55	59	65	77
1.25	56	70	73 (70)	80 (71)	75 (74)
2.5	55	72 (71)	71	74 (72)	85 (75)
5.0	63	61 (74)	71 (74)	75	84 (79)
10.0	60	65 (86)	81 (87)	74 (87)	91

[a] Values represent mean migration distances (μm) of triplicate determinations; the standard deviation in all cases was less than 10%. Numbers in parentheses are calculated distances expected when migration is due only to enhanced random movement (21).

A checkerboard assay was performed with CF to determine whether the multiwell plate assay was able to discriminate between chemokinesis and chemotaxis (Table 1). For wells above the diagonal in Table 1, which had a higher concentration of agent below the filter (a positive chemotactic gradient), experimental values exceeded the results calculated by assuming migration was due only to chemokinesis. Conversely, the presence of a negative gradient produced values lower than expected. These data indicate that the multiwell plate method measured true chemotactic activity, and PMN chemotaxis data reported below were obtained with this method.

Production of chemotactic factor. The growth of *B. dermatitidis* was less in TC199 than in CBM, which contained 10 times more glucose (Fig. 3). The logarithmic phase of growth was more extensive in CBM; the stationary phase of growth was reached by 10 days in TC199 and by 21 days in CBM. Mean generation times (dry weight doubling times) during the period of logarithmic growth were calculated to be 5.7 days in TC199 but only 1.9 days in CBM. The initial inoculum was almost entirely in the yeast morphology and remained so throughout the growth period in both media. Clumping of yeast cells increased as the growth in each medium reached the stationary phase; clumping was especially marked in CBM after approximately 21 days (as reflected by the reduction in direct counts of CFU per ml; Fig. 3, arrow). Yeast cell clumps appeared to reflect the failure of budded cells to detach, rather than the aggregation of separate cells. Measurement of pH and chemotactic activity of CFs revealed that the pH of TC199 rose to 9 and that medium produced maximal chemotactic activity (Fig. 4). No chemotactic activity was detectable in CBM, and the pH of this medium dropped to about 6. In subsequent investigations of different media, it was determined that culture filtrates of both TC199 and MEM possess optimal chemotactic activity for PMNs at concentrations of 10% (vol/vol) by approximately 17 days of incubation (Table 2). Culture filtrates of CBM-PB at pH 6.0 had no chemotactic activity. Some chemotactic activity was detectable in CBM-PB at pH 7.2; however, the activity was less than that obtained with either TC199 or MEM. Production of chemotactic activity did not correlate with the amount of growth (Fig. 3 and 4). The pHs of TC199 and MEM CFs increased to 9.0 and 9.2, respectively, and remained alkaline over the period of incubation. In CBM-PB the pH of the medium remained essentially unchanged at 6.0 or 7.2. All subsequent results are reported for MEM-derived CF.

Monocyte chemotaxis. Blind-well chambers were used to evaluate monocyte chemotaxis to various chemotaxins, including CF. Monocytes migrated toward a 10% (vol/vol) dilution of CF in a degree similar to that of the chemotaxis to other test agents (Table 3).

Protein and carbohydrate analysis. A 10-fold concentration of dialyzed CF had an estimated protein content of 8 μg/ml; total hexoses and pentoses in concentrated CF were 600 μg/ml.

pH and temperature stability of the factor. Chemotaxis assay of CF incubated at various pHs for 18 to 24 h

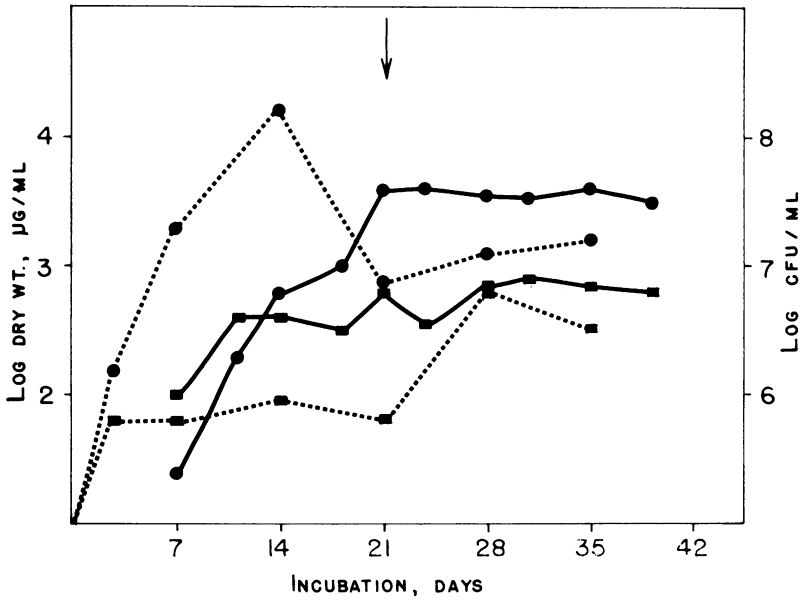

FIG. 3. Growth of *B. dermatitidis* ATCC 26199 at 37°C in TC199 (■) and CBM (●). Cultures were initiated with 10^5 primed yeast cells per ml and sampled periodically for growth, which was quantified by dry weight (——) and hemacytometer counts (CFU/ml) (· · ·). The arrow indicates the time at which the yeast cells formed appreciable clumps in CBM (see text).

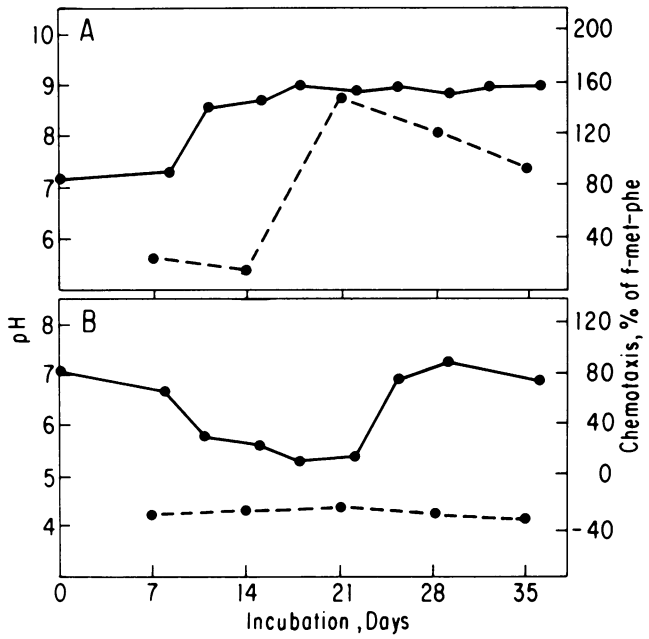

FIG. 4. Chemotactic activity for neutrophils and pH of CFs of *B. dermatitidis*. Cultures of TC199 (A) and CBM (B) inoculated at 10^5 yeast cells per ml were sampled periodically for chemotactic activity (-----) and pH (———). Chemotaxis of 10% (vol/vol) dilutions of CF is expressed as the mean percentage of the PMN migration distance toward 2 μM *N*-formyl-L-methionyl-L-phenylalanine (f-met-phe).

demonstrated a significant decrease in chemotactic activity for PMNs after incubation below pH 7 (Table 4). Incubation at pH 3, 4, or 5 also abolished chemotactic activity (data not shown); the destruction of chemotactic activity by acid treatment was irreversible as late as 48 h after neutralization. Exposure to pH 9 or 10 had no discernible effect on chemotactic activity in CF (data not shown). Neither three cycles of freezing and thawing nor incubation at 56 or 100°C for 60 min caused any apparent modification in chemotactic activity, as compared with that of an untreated control.

Deactivation of chemotactic response. PMNs were preincubated with either f-MLF or CF to determine whether the response of treated cells to fresh chemotaxin was decreased. PMNs assayed for chemotactic responsiveness to each agent, as well as to ZAS and HBSS-BSA, indicated that f-MLF deactivated chemotaxis to f-MLF and to CF (Fig. 5). The converse, however, was not true. Preincubation of PMNs with CF caused homologous deactivation only. Preincubation with either f-MLF or CF did not deactivate the PMN chemotactic response to ZAS. Similar results were obtained in three experiments in which different batches of PMNs were used.

Adsorption of chemotaxin. After adsorption with Formalin-killed PMNs, CF was assayed for activity at both 10 and 20% (vol/vol) dilutions. The results were similar with both sets of dilutions, and data for the 10% adsorbed CF are shown in Table 5. Incubation of CF with 10^6 PMNs per ml significantly decreased chemotactic activity. Incubation with 10^7 PMNs per ml reduced chemotactic activity nearly to the level of the negative control HBSS-BSA. Similar results were obtained with PMNs having a viability of >95% at the conclusion of the experiment (data not shown).

DISCUSSION

CFs of *B. dermatitidis* grown in TC199 or MEM yielded high levels of chemotactic activity for neutrophils (Fig. 2 and 3 and Table 2). Little chemotactic activity for PMNs was detected in CFs from CBM-PB at pH 7.5, and none was detected in CFs from CBM buffered to pH 6.0. We therefore concluded that chemotaxin production is not medium dependent but may be sensitive to acidic conditions. Experiments with pH adjustment supported this conclusion, as activity was irreversibly destroyed by exposure of chemotactic filtrates to pH values below 7 (Table 4). In cultures grown in media such as CBM, a chemotactic factor may be produced but inactivated soon after its elaboration by acidity produced during glucose metabolism. CBM-PB has 10 times the available glucose of TC199 and MEM, which may account for the increased growth in and the drop in pH of this medium (Fig. 3 and 4). The rather alkaline pH of older TC199 or MEM cultures had no effect on their chemotactic activities, and CFs adjusted to pH 10 and incubated for 24 h showed no significant changes in chemotactic activity. MEM was selected for subsequent production and evaluation of *B. dermatitidis* CFs because its composition and support of chemotaxin production were similar to those of TC199 (12) and because, unlike TC199, MEM was not contaminated with endotoxin (as determined by the *Limulus* amoebocyte lysate assay). Endotoxin was of concern because its presence may nonspecifically stimulate random migration.

Preincubation of viable PMNs with chemotactic factors has been reported to reduce the subsequent chemotactic responsiveness of those cells to chemotaxins and to nonspecifically decrease spontaneous locomotion. Receptor deactivation studies have established that the receptors for ZAS differ from those of the formylated peptides (8, 13, 15).

TABLE 2. Neutrophil chemotaxis to *B. dermatitidis* CFs in various media

Medium	pH on day: 0	pH on day: 17	Chemotaxis (μm)[a]	P[b]
TC199	7.2	9.0	112 ± 3	<0.005
MEM	7.4	9.2	111 ± 2	<0.005
CBM-PB	6.0	6.0	60 ± 2	NS
CBM-PB	7.5	7.2	86 ± 6	NS

[a] Mean of triplicate determinations ± standard deviation; all samples were assayed at 10% (vol/vol) dilutions in HBSS-BSA. Random migration in HBSS-BSA was 60 ± 5 μm.

[b] Statistical significance of migration distances was evaluated by the Student one-sided *t* test. Each agent was compared with the random migration control HBSS-BSA; *P* values are shown. NS, Not statistically significant ($P \geq 0.10$).

TABLE 3. Comparison of human monocyte chemotaxis to *B. dermatitidis* CF and other chemotaxins

Agent[a]	Chemotaxis (μm)[b]	P[c]
HBSS-BSA	100 ± 3	
CF	165 ± 6	<0.0025
BF	146 ± 5	<0.0025
f-MLF	158 ± 6	<0.0025
ZAS	167 ± 5	<0.0025

[a] HBSS-BSA was the diluent control. Chemoattractants were 10% (vol/vol) *B. dermatitidis* CF, 10% (vol/vol) bacterial factor (BF) 20 nM f-MLF, and 1% (vol/vol) ZAS.

[b] Mean of triplicate determinations ± standard deviation.

[c] See Table 2, footnote *b*.

TABLE 4. Chemotactic activity for neutrophils in *B. dermatitidis* CF incubated for 18 h at various pHs

pH	Chemotaxis (μm)[a]	P[b]	% Control activity[c]
7.4	107 ± 5	NC[d]	102
7.2	106 ± 6	NC	100
7.0	108 ± 8	NC	104
6.8	88 ± 8	<0.05	63
6.6	92 ± 8	<0.05	71
6.4	96 ± 9	<0.15	79
6.2	84 ± 4	<0.0125	54
6.0	87 ± 7	<0.0125	60

[a] Mean of triplicate determinations ± standard deviation; all samples were assayed at 10% (vol/vol) dilutions in HBSS-BSA.
[b] Migration to untreated CF control used for statistical analysis 106 ± 5 μm; see Table 2, footnote *b*.
[c] Expressed as: (100) (sample migration distance − HBSS-BSA random migration)/(untreated CF migration distance − HBSS-BSA random migration), where HBSS-BSA random migration was 58 ± 7 μm.
[d] NC, Not calculated.

TABLE 5. Adsorption of chemotactic activity for neutrophils from dialyzed *B. dermatitidis* CF with Formalin-killed neutrophils

Preincubation (PMN/ml)[a]	Chemotaxis (μm)[b]	P[c]
None	64 ± 2	
10^5	58 ± 4	NS
10^6	56 ± 1	<0.0125
10^7	51 ± 5	<0.05
10^8	51 ± 4	<0.025

[a] Final density of PMNs in CF during preincubation.
[b] Mean of triplicate determinations ± standard deviation; random migration in HBSS-BSA alone was 48 ± 1 μm. All samples were assayed at 10% (vol/vol) dilutions in HBSS-BSA.
[c] Statistical significance of each adsorbed CF sample was compared with CF incubated without PMNs; see Table 2, footnote *b*. NS, Not significant.

Exposure of PMNs to high concentrations of f-MLF induces prompt, specific deactivation and a delayed, nonspecific decrease in chemokinesis (8, 13). Functional loss of receptors after exposure to chemotaxin results in PMNs which are no longer stimulated by this chemotaxin but which may retain the capacity to respond to a chemotaxin specific for another receptor (15). In this study, the responses of PMNs to f-MLF and CF were specifically deactivated after preincubation, whereas chemotaxis to ZAS was unaffected (Fig. 5), indicating that deactivation was preferential (8). Deactivation with f-MLP also reduced the chemotactic response to CF. These data suggest that the receptor for the *B. dermatitidis* factor also has affinity for f-MLF. It is also possible that both chemotaxins bind to separate receptors, then either compete metabolically (perhaps by stimulation of oxidative metabolism [13]) or have dissimilar receptor recycling. Differences in the affinities of CF and f-MLF for receptor binding may explain the lack of reciprocal deactivation. Binding studies which directly address this question are in progress.

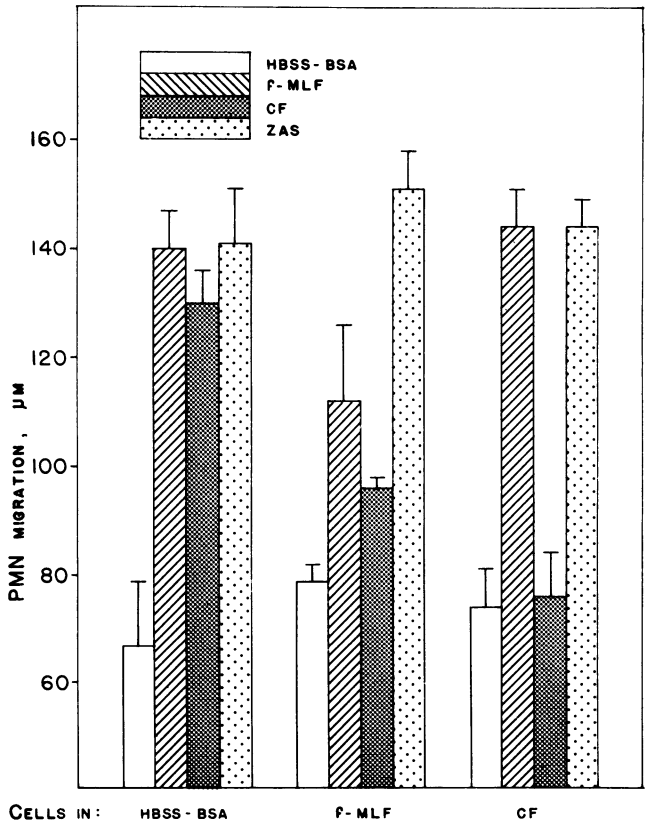

FIG. 5. Deactivation of neutrophil chemotaxis. PMNs were incubated with either f-MLF or dialyzed, concentrated *B. dermatitidis* CF at final concentrations 100-fold greater than chemotactic optimum or in diluent HBSS-BSA alone. PMNs in each group were then washed and assayed for chemotactic response to 20 nM f-MLF, 10% (vol/vol) CF, 1% (vol/vol) ZAS, and HBSS-BSA. Bars indicate mean migration distances of triplicate determinations; standard deviations are indicated by vertical lines.

As indicated above, the evalution of chemotaxis assumes that certain events occur before and after the observation of chemotactic movement. Initially, chemotaxin binds to a specific receptor on the cell surface and becomes internalized; the cell is then metabolically activated for locomotion. Since the chemotactic factor in CF is thus far detectable only by biological assay, binding of the factor to PMNs to specifically remove the activity was tested. Samples of CF were adsorbed with viable or Formalin-killed PMNs, before being assayed for chemotactic activity for fresh PMNs, to determine whether binding of the factor to neutrophil receptors would occur with sufficient avidity and number to adsorb the chemotaxin. Preincubation with 10^6 PMNs provided adequate receptors to remove a significant portion of the chemotactic activity from 1 ml of CF (Table 5); alternatively, the chemotaxin may have been modified to a less active form.

The observation that monocytes were also strongly attracted to CF (Table 3) is compatible with the histopathological accumulation of mononuclear cells and PMNs at infective foci. The distinctive infiltration of PMNs in blastomycotic lesions may be stimulated by the elaboration of a serum-independent chemotactic factor and augmented by the generation of endogenous chemotaxins. This possibility is currently under investigation.

The use of inexpensive, disposable multiwell plates for chemotaxis assays circumvents the problems associated with the manipulation of blind-well chambers and permits the assay of larger numbers of samples. This method also uses one-third fewer cells. Readily available membrane filters require no cutting or other alteration. Less time is needed to perform the multiwell plate chemotactic assay, because several steps required by the chambers are eliminated, such as aspiration of the agent and cells from the upper

wells, manipulation of the retaining caps, and washing the reuseable chambers and caps. The problem of accumulation of residual chemotactic agents in reuseable chambers is completely avoided. Since chemotaxis in the multiwell plate system decayed after 45 min, the optimal time for incubation was set at 35 min (Fig. 2). This interval provided excellent correlation between the two methods (Fig. 3) and yielded comparable between-run coefficients of variation. The multiwell plate method was further validated by results of checkerboard assays, which indicated that the PMNs responded to increasing chemotactic gradients and to increasing amounts of the chemotaxin. Values for migration in the presence of a positive gradient were greater than those expected from chemokinesis alone (Table 1). The assay of chemotactic response of neutrophils by the multiwell plate system is efficient and economical. The method is simple, easy to set up, and adaptable to a variety of laboratory situations.

B. dermatitidis is a slow-growing pathogen and reaches stationary phase only after about 3 weeks of incubation, at which time factor production is detectable. Maximum amounts of chemotaxin were produced in media more closely approximating physiological conditions, i.e., TC199 and MEM, which contain only 0.1% (wt/vol) glucose, versus CBM, which has 1.0% (wt/vol) glucose. The notably small amount of protein detected in CF was consistent with the observation that little autolysis of the yeast cells occurred during this incubation period. The chemotactic activity of CF was remarkably stable to heating and to repeated freeze-thaw cycles. It is also possible, though unlikely, that such treatments actually destroyed and inactivated the factor and that subsequent aggregation generated novel chemotaxins with comparable activity. The factor may be a carbohydrate or glycoprotein, as significant amounts of hexose-pentose were detected in dialyzed CF. The composition, an unusual primary or secondary peptide structure, or size of the factor may contribute to its thermostability. Lipid chemotaxins from microbial CFs have also been reported (20), and the physical characteristics of CF are compatible with this possibility. Current investigations involve clarification of the biochemical nature of this chemotaxin.

The production of a chemotactic factor by yeast cells of *B. dermatitidis* may be related to the appearance in vivo of a significant accumulation of PMNs (and macrophages) at sites of infection and inflammation. The demonstration of the in vitro production of a chemotaxin and its stability to high but not low pH may provide a partial explanation for this response. Chemotaxin might be elaborated during infection, but the development of an acidic environment in the tissue after suppuration would destroy the activity of the factor. By that time, sufficient PMNs would have accumulated to be further attracted by the continuing production of host chemotactic factors. Macrophage accumulation around the foci might also be prolonged in this manner. Further investigations into the biochemistry and biological activity of this factor in vivo intend to further clarify the host-fungus interaction in blastomycosis. Variations in chemotaxin production among *B. dermatitidis* strains may correlate with differences in pathogenicity (4).

ACKNOWLEDGMENTS

We thank Stephen R. Turner for helpful discussions regarding applications of the chemotaxis methods and generous technical advice. The expert typing and administrative skills of Dayle Wilkins are greatly appreciated.

This research was suported in part by a grant-in-aid of research sponsored by Sigma Xi, the Scientific Research Society, and by a grant from the Walker P. Inman Fund. L.M.T. was supported by Infectious Diseases training grant 5T32-AI07148.

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