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Mechanisms of Fungal Pathogenicity: Correlation of Virulence In Vivo, Susceptibility to Killing by Polymorphonuclear Neutrophils In Vitro, and Neutrophil Superoxide Anion Induction among *Blastomyces dermatitidis* Isolates

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Seven *Blastomyces dermatitidis* isolates varying in virulence for mice were compared for susceptibility to polymorphonuclear neutrophil (PMN) killing and the ability to induce superoxide anion (O_2^-) production by PMNs in vitro. In vitro killing of six *B. dermatitidis* isolates by murine peripheral blood PMNs or by PMNs elicited from the peritoneal cavity by a local immune reaction (*B. dermatitidis*-immune mice given killed *B. dermatitidis* intraperitoneally 24 h earlier) inversely correlated with in vivo virulence (most to least virulent isolates: VV, V, V40, KL-1, A2, and GA-1). The capacity of isolates to induce O_2^- production by PMNs also inversely correlated with in vivo virulence. Isolate A, of intermediate in vivo virulence, was a good inducer of O_2^- production in vitro but was no more susceptible to in vitro killing by PMNs than isolate V, VV, or V40. Fungal intracellular superoxide dismutase or catalase content did not correlate with in vivo virulence or in vitro killing by PMNs. Isolate A, however, had two to four times the intracellular catalase activity as did other *B. dermatitidis* isolates, suggesting a possible mechanism for its enhanced resistance to in vitro killing by PMNs. Therefore, while in vitro killing by PMNs and the capacity to induce O_2^- production by PMNs inversely correlated with virulence for six *B. dermatitidis* isolates, isolate A was an exception: its resistance to killing by PMN-generated oxygen metabolites in vitro but its susceptibility to killing in vivo suggest that its in vivo killing occurs by other, perhaps nonoxidative, mechanisms.

Blastomyces dermatitidis is a thermally dimorphic fungal pathogen which occurs in tissue as a broad-based budding yeast. Several research groups have attempted to define factors which contribute to the virulence of this organism by comparing virulent, attenuated, or avirulent strains for differences in lipid content (4, 5, 11, 16), cell wall composition (11, 12), ability to multiply in vivo (7), or ability to evade killing in vitro by polymorphonuclear neutrophils (PMNs) (37) or macrophages (7). Despite these efforts, little is known about the pathogenic mechanisms of this organism.

Histopathological studies suggest a role for macrophages and PMNs in disease caused by *B. dermatitidis* (25). It has been shown that macrophages and PMNs can be activated in vitro (8–10, 27) or in vivo (6, 28, 29) to kill *B. dermatitidis*. It has also been documented that oxidative mechanisms are important in the killing of *B. dermatitidis* by PMNs (17, 29, 30) and that this fungus is sensitive to the oxygen metabolites known to be produced by PMNs (35, 36).

A murine model of pulmonary blastomycosis (20) makes it possible to address certain questions concerning virulence. We used it to compare the in vivo virulence of seven *B. dermatitidis* strains to their susceptibility to killing in vitro by PMNs from two sources (peritoneal cavity and peripheral blood), their capacity to induce the production of superoxide anion by PMNs in vitro, and their intra- and/or extracellular content of superoxide dismutase (SOD) and catalase.

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MATERIALS AND METHODS

Animals. Specific-pathogen-free BALB/cByJIMR male mice (Institute for Medical Research, San Jose, Calif.) 8 to 10 weeks of age were used.

Fungi. Inocula of *B. dermatitidis* were prepared for in vivo or in vitro challenge by harvest of 48- to 72-h growth from blood agar plates (BAP; BBL, Cockeysville, Md.) incubated at 35°C. Fungal cells were washed, and appropriate dilutions were plated in triplicate on BAP to determine the number of CFU per milliliter. *B. dermatitidis* isolates used for this study were as follows: V (ATCC 26199), a patient isolate deposited at the American Type Culture Collection in 1970, received in our laboratory in 1984, and stored on brain heart infusion (BHI) slants under water at 4°C; VV, ATCC 26199 obtained from ATCC in 1986 and stored on BHI slants under water at 4°C; V40, originally ATCC 26199 but passaged 40 times on BHI agar at 35°C; A (ATCC 60915), originally ATCC 26199 but converted to an attenuated variant by serial passages on BHI agar at 35°C for 1.5 years (5); A2 (ATCC 60916), originally ATCC 26199 but spontaneously converted to avirulence after storage at −20°C for 1.5 years; KL-1 (ATCC 26198), originally a virulent strain when isolated from the soil as reported in 1961 (15) and 1964 (14) but subsequently showing a loss of virulence for mice (5) and presently showing avirulence in the isolate stored at the American Type Culture Collection (10a); and GA-1 (ATCC 26197), an avirulent strain originally isolated from a human with chronic cutaneous blastomycosis (11). Isolates A and A2 have retained attenuated virulence and susceptibility to in vitro killing for over 4 years. Mice have been infected with isolate A or A2, and each isolate has been recovered from

the animals. Reversion of these isolates to the virulence phenotype has not been seen after such animal passage.

In vitro rates of growth of isolates in liquid fungal medium or tissue culture medium with or without mouse serum, mouse lung homogenate, or mouse lung washings were shown (4, 5) not to correlate with virulence, and current studies with isolate A2 confirmed these observations.

Reagents and media. Complete tissue culture medium consisted of RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 100 U of penicillin, and 100 μg of streptomycin per ml. These and other reagents, including preservative-free ammonium heparin, catalase, SOD, Hypaque 1077, dextran (molecular weight, 250,000), and metrizamide, were purchased commercially as described previously (29, 30).

Immunization of mice. Mice were inoculated with 5,000 CFU of *B. dermatitidis* yeast cells intradermally at two dorsal sites. These mice were referred to as immune mice after the resolution of the infection over a 4-week period (26).

Infection of mice. Mice were anesthetized with ether, and 0.03 ml of *B. dermatitidis* suspended in 0.85% saline was instilled intranasally per mouse (20). When mice were infected by the intraperitoneal route, yeast cells were administered in 0.1 ml of 0.85% saline per mouse. Mice were observed daily for mortality over a period of 60 days.

PMNs. Normal peripheral blood PMNs (PB-PMNs) and mononuclear cells (PBMC), obtained by axillary bleeding of mice, were purified by Ficoll-Hypaque separation as previously described (29); PB-PMNs were further purified by dextran sedimentation (29) and hypotonic lysis of contaminating erythyrocytes (27).

Peritoneal exudate cell PMNs (PE-PMNs) were elicited from *B. dermatitidis*-immune mice by injection of 500 μg (wet weight) of nonviable yeast cells in 0.5 ml of 0.85% saline intraperitoneally 24 h prior to cell harvest. PE-PMNs were collected by repeated lavage of the peritoneal cavity and purified by metrizamide gradient centrifugation as described previously (30).

Cocultures of PMNs and *B. dermatitidis*. Purified PB-PMNs (89% PMNs, 9% lymphocytes, and 2% monocytes, as determined with Wright-Giemsa-stained smears) or purified PE-PMNs (87% PMNs, 9% lymphocytes, and 4% monocytes) were suspended in complete tissue culture medium (5×10^6/ml) and dispensed (0.1 ml per well) into 96-well Micro Test III plates (Becton Dickinson, Oxnard, Calif.) for fungal killing assays. PMNs were challenged with 0.1 ml of *B. dermatitidis* yeast cells (10^4 CFU/ml) and 0.02 ml of fresh normal mouse serum per well. Triplicate or quadruplicate cocultures were incubated for 2 h at 37°C in 95% air–5% CO_2. After incubation, cocultures were harvested with distilled water as described previously (9), diluted in 0.85% saline, and plated on BAP for the determination of CFU per well. The percent reduction of inoculum CFU was calculated as follows: [1 − (coculture CFU/inoculum CFU)] × 100. It has been observed that *B. dermatitidis* yeast cells are too large for individual phagocytes to engulf (8, 27). PMNs cluster around the yeast cells, and killing occurs by what is therefore assumed to be an extracellular process.

Determination of superoxide anion production by PMNs. Superoxide anion (O_2^-) production was quantitated in a ferricytochrome *c* reduction assay with freshly opsonized viable *B. dermatitidis* yeast cells as the inducing stimulus. Opsonized yeast cells were prepared by incubating 2×10^7 yeast cells in 1 ml of fresh normal mouse serum for 30 min at 37°C in a shaking water bath prior to use. The viability of yeast cells was retained during the opsonization procedures. PMNs (7.0×10^5 per test tube) were incubated with the following components for 30 min in a shaking water bath at 37°C (1) to allow for optimal ferricytochrome *c* reduction: ferricytochrome *c* (40 μM), yeast cells (4×10^6/ml), and fresh normal mouse serum (2 to 4%). The final volume in each tube was 2.5 ml. Background values for samples containing opsonized *B. dermatitidis* cells but no PMNs did not vary significantly between isolates, nor did they correlate with virulence; they were subtracted from values for samples containing PMNs (mean background O_2^- production: virulent isolates V and V40, 5.7 ± 2.6 nmol/ml; isolates A, KL-1, A2, and GA-1, 4.8 ± 3.4 nmol/ml). Selected tubes received SOD (3,000 U/ml); subtraction of values obtained for these samples from those obtained for test samples without SOD yielded the SOD-inhibitable reduction of ferricytochrome *c*. Following incubation at 37°C, test tubes were placed on ice and cleared by centrifugation at 1,000 × *g*, and supernatants were read spectrophotometrically at 550 nm. Ferricytochrome *c* reduction was determined with an extinction coefficient of $2.1 \times 10^4\ M^{-1}\ cm^{-1}$ (22).

Determination of fungal intracellular catalase and SOD activities. *B. dermatitidis* yeast cells were grown in SAAMF broth, a defined synthetic medium for fungal culturing (21). For each isolate tested, 200 ml of SAAMF was inoculated with 10^7 CFU and placed on a rotary shaker (140 rpm) in air at 35°C for 72 h.

Fungal cells were collected by centrifugation (1,000 × *g*) at an ambient temperature. Supernatants were decanted and filter sterilized. Pelleted cells were washed by centrifugation in ice-cold 0.05 M potassium phosphate buffer (pH 7.0) containing 10^{-4} M EDTA. Yeast cells were resuspended in ice-cold buffer at a ratio of 1 ml of yeast cells to 2.5 ml of buffer, and the suspension was delivered into 15-ml conical test tubes on ice and containing 1.5 ml of ethanol-washed, oven-dried glass beads (0.45- to 0.50-mm diameter; Glasperlen; B. Braun Melsungen AG, Melsungen, Germany). Tubes were vortexed for eight 15-s periods, with a 45-s interval on ice between each 15-s period of vortexing. Microscopic examination showed that greater than 97% of the cells were disrupted by this method. Broken cell suspensions were centrifuged at 1,700 × *g* for 5 min at 4°C. Supernatants were collected and ultracentrifuged for 30 min at 218,000 × *g* and 4°C. Supernatants were assayed immediately for released enzyme activities.

Catalase activity was assayed by the method of Beers and Sizer (3) by monitoring the disappearance of reagent hydrogen peroxide by measuring the decrease in the A_{240} at 25°C over a period of 2 to 3 min. Bovine liver catalase (Sigma Chemical Co., St. Louis, Mo.) was used as the standard; 1 U was defined as the decomposition of 1.0 μmol of H_2O_2 per min at pH 7.0 and 25°C.

Intracellular SOD activity for *B. dermatitidis* isolates was assayed by the SOD-inhibitable reduction of ferricytochrome *c* with the xanthine-xanthine oxidase system of Crapo et al. (13) and bovine erythrocyte SOD (Sigma) as the standard. Extracellular SOD activity was not detected.

Protein determination. The protein content of samples was determined by the method of Lowry et al. (24).

Statistical analyses. The Student *t* test was used for comparisons between groups for in vitro experiments. Means are expressed as plus or minus the standard deviation from the mean. The Wilcoxon test was used for comparisons between groups for in vivo experiments. Pearson's *r* value was used for determinations of correlation. Significance was desig-

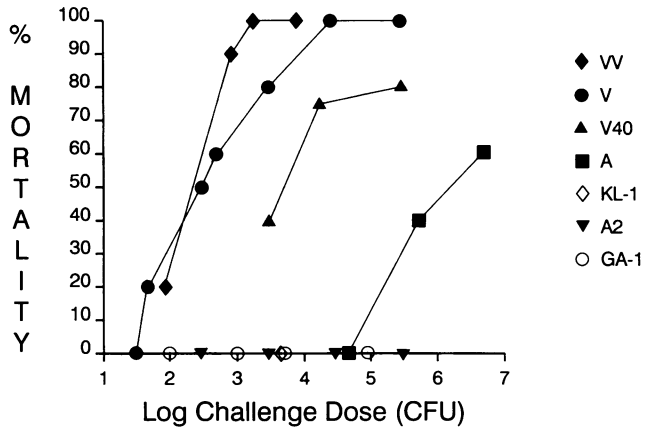

FIG. 1. Comparison of mouse mortality after intranasal challenge with *B. dermatitidis* isolates. Normal adult mice (8 to 25 per group) received the number of CFU indicated and were observed for mortality over a period of 60 days.

nated as $P < 0.05$. The statistical methods used were those described by Edwards (18).

RESULTS

Comparative virulence of *B. dermatitidis* isolates in mice infected by the intranasal route. Intranasal challenge of adult (>25-g) mice demonstrated significant differences in the virulence of the *B. dermatitidis* isolates tested (Fig. 1). The 50% lethal doses (LD_{50}s) for the most virulent isolates, VV, V, and V40, were $10^{2.3}$, $10^{2.6}$, and $10^{3.5}$, respectively. The LD_{50} for isolate A (a mutant induced by serial passages of parent isolate V) was $10^{6.2}$, and there was no observed mortality with any other isolates tested over a wide range of challenge doses (Fig. 1). Isolates V40, V, and VV are therefore 500, 4,000, and 8,000 times more virulent, respectively, than isolate A. A challenge dose of 4.8×10^4 CFU of isolate A resulted in no deaths by day 60, while lower doses of 8.3×10^3 CFU of isolate VV or 2.6×10^4 CFU of isolate V resulted in 100% mortality and 3×10^4 CFU of isolate V40 resulted in 75% mortality ($P < 0.01$). Intranasal challenge with 3×10^5 CFU of isolate V40 resulted in twice the mortality (80%) as did 4.8×10^5 CFU of isolate A ($P < 0.01$).

An intranasal challenge dose as high as 3×10^5 CFU of isolate A2 (a spontaneous mutant of parent isolate V) proved nonlethal ($P < 0.01$ or $P < 0.05$ for A2 versus VV or V at all concentrations tested above 500 CFU and versus V40 at all concentrations above 3,000 CFU). Results similar to those obtained for A2 were obtained for KL-1 and GA-1 (Fig. 1). The low virulence of isolates KL-1 and GA-1 is supported by previous studies in which an intranasal inoculum of $10^{5.9}$ CFU of isolate KL-1 (5) and an intraperitoneal inoculum of $10^{5.6}$ CFU of isolate GA-1 (4) were nonlethal.

Differences in virulence could not be explained by mechanical differences in the capacity of isolates to enter the lungs, for two reasons. First, when isolates V and A2 were given by the intraperitoneal route (a route by which all organisms reach the site of infection) rather than by the intranasal route, V continued to be more virulent than A2. Isolate V (3,000 to 6,000 CFU per mouse) given intraperitoneally resulted in 80% mortality, while 10,000 CFU of isolate A2 was nonlethal ($P < 0.01$). Necropsy of survivors revealed no gross organ pathology in 100% of mice injected with A2.

TABLE 1. Comparative in vitro killing of *B. dermatitidis* isolates by PMNs

B. dermatitidis isolate	Mean ± SD % killed (no. of experiments) by[a]:	
	PE-PMNs	PB-PMNs
V	51.7 ± 12.4 (6)	38.0 ± 14.9 (10)
VV	60.7 ± 15.3 (2)	ND
V40	62.1 ± 16.1 (2)	42.3 ± 19.3 (3)
A	58.9 ± 17.3 (6)	47.7 ± 15.0 (8)
KL-1	85.6 ± 12.6 (4)[b]	68.4 ± 15.1 (4)[b]
A2	80.2 ± 12.7 (7)[b]	69.1 ± 13.3 (4)[b]
GA-1	90.3 ± 7.5 (6)[b]	97.5 ± 1.0 (4)[b]

[a] PE-PMNs from *B. dermatitidis*-immune mice or PB-PMNs from untreated mice were cocultured with the indicated *B. dermatitidis* isolate for 2 h, and the reduction in inoculum CFU was determined as described in Materials and Methods. ND, not determined.

[b] Isolates KL-1 ($P < 0.005$), A2 ($P < 0.005$), and GA-1 ($P < 0.001$) were significantly more susceptible to killing by PE-PMNs or PB-PMNs than was isolate V.

In contrast, surviving mice infected with isolate V showed evidence of peritoneal abscesses as well as liver and spleen involvement. No death or histopathological evidence of fungal disease was observed when mice were given 10^7 CFU of isolate A2 intranasally in addition to 10^6 CFU of isolate A2 intraperitoneally and were examined at intervals of up to 14 days postinoculation.

Previous studies also demonstrated the greater virulence of isolate V than of isolate A or GA-1 when mice were infected by the intraperitoneal route (4, 5). This was also true after infection by the subcutaneous route (7). These differences in isolate virulence were more pronounced between isolates V and GA-1 than between isolates V and A (4, 5).

Second, lungs of intranasally infected mice were removed at intervals following infection with 1.8×10^5 CFU of isolate A2, homogenized, and plated on BAP for colony formation. Eight hours after infection, 80.6% of the original inoculum was recovered from infected lungs. By day 4 postinfection, recoverable CFU from the lungs had dropped to 0.9% of the original inoculum. These data demonstrate that at least 80% of the fungal inoculum entered the lungs but that isolate A2 was cleared without mortality.

Comparative in vitro killing of *B. dermatitidis* isolates by PMNs. We compared the susceptibility of *B. dermatitidis* isolates to in vitro killing by PB-PMNs or PE-PMNs (28, 30). In vitro killing of yeast cells by PMNs from either source correlated inversely with in vivo virulence of *B. dermatitidis* isolates (Table 1). High-virulence isolates V, VV, and V40 were the most resistant to in vitro killing by PMNs, while low-virulence isolates KL-1, A2, and GA-1 were killed more effectively (Table 1). Of the virulent isolates, isolate V was the prototype selected for sufficient studies for statistical purposes (although VV, in the few experiments performed, was also significantly different from GA-1 and KL-1 in percent killing). Although isolate A was less virulent than V, VV, or V40 in vivo, there was no detectable difference between isolate A and the more virulent isolates in susceptibility to in vitro killing by PMNs. These findings indicate that isolate A has a relative resistance to the mechanisms used by PMNs to kill fungi and suggests that it is killed more effectively than the virulent isolates in vivo by host defense mechanisms other than those of PMNs. The Pearson's *r* values comparing isolate LD_{50}s with in vitro killing by PE-PMNs were as follows: including isolate A, 0.81 ($P < 0.005$); excluding isolate A, 0.95 ($P < 0.005$). The Pearson's

TABLE 2. Induction of PMN superoxide anion production by *B. dermatitidis* isolates

B. dermatitidis isolate	Mean ± SD nmol of O_2^-/10^6 PMNs/h (no. of experiments)[a]
V	1.2 ± 1.6 (3)
V40	2.0 ± 2.0 (2)
A	23.6 ± 7.2 (4)[b]
KL-1	11.2 ± 7.2 (3)[b]
A2	26.4 ± 9.6 (5)[b]
GA-1	15.0 ± 5.8 (2)[b]

[a] PB-PMNs were mixed with freshly opsonized *B. dermatitidis* yeast cells of the indicated isolate, and O_2^- production was determined in a ferricytochrome *c* reduction assay as described in Materials and Methods.

[b] Isolates A, KL-1, A2, and GA-1 induced significantly ($P < 0.05$) more superoxide anion production from PMNs than did isolate V. Isolate VV was not tested.

r values comparing isolate LD_{50}s with in vitro killing by PB-PMNs were as follows: including isolate A, 0.78 ($P < 0.005$); excluding isolate A, 0.87 ($P < 0.005$). LD_{50}s for these comparisons were not experimentally obtained for A2, KL-1, and GA-1 (since no deaths occurred with the inocula tested) and were therefore assigned as the highest dose of *B. dermatitidis* yeast cells (10^7) that could be given intranasally.

Induction of PMN superoxide anion production by *B. dermatitidis* isolates. We chose to determine possible mechanisms for the observed differences in isolate susceptibility to in vitro killing by PMNs. Documented mechanisms by which PMN kill microorganisms include those involving the production of active oxygen species, such as superoxide anion (O_2^-), H_2O_2, and hydroxyl radical (·OH) (2, 23). We previously demonstrated that isolates of *B. dermatitidis* were susceptible to in vitro killing by PMN metabolites of oxygen (30, 35, 36). We chose therefore to examine whether the ability of *B. dermatitidis* isolates to induce the production of superoxide anion by PB-PMNs in in vitro cocultures correlated inversely with in vivo virulence.

High-virulence isolates V and V40 were poor inducers of PMN O_2^- production (Table 2). In contrast, low-virulence isolates KL-1, A2, and GA-1 induced PMNs to release 10- to 20-fold more O_2^- than did isolate V (Table 2). Although intermediate in in vivo virulence, isolate A induced PMN O_2^- production as well as did the low-virulence isolates. These data indicate that the capacity of *B. dermatitidis* isolates to induce the production of O_2^- by PB-PMNs in vitro correlates inversely with in vivo virulence (Pearson's *r* values for LD_{50}s versus O_2^- production in vitro: including isolate A, 0.81 [$P < 0.005$]; excluding isolate A, 0.84 [$P < 0.005$]). Isolate A, therefore, has a unique relative resistance to in vitro killing by PMNs which corresponds to a resistance to the effects of the O_2^- that it induces in PMNs.

Comparison of fungal catalase and SOD levels with in vivo virulence. To further examine the mechanisms responsible for the correlation of isolate susceptibility to in vitro killing with in vivo virulence and to further elucidate the unique resistance of isolate A to killing by PMNs despite its significant induction of O_2^- production by PMNs, we compared the intracellular and extracellular contents of fungal enzymes reported to protect microbes from oxidative damage by phagocytic cells (32, 35): SOD, which removes O_2^- anions by converting them to H_2O_2 (13, 23), and catalase, which catalyzes the breakdown of H_2O_2 to H_2O and O_2 (3).

B. dermatitidis isolates did not show any significant differences in the intracellular content of SOD (mean units per milligram of protein plus or minus the standard deviation from the mean for the number of experiments shown in brackets: V, 2.8 ± 2.5 [5]; A, 1.8 ± 3.1 [3]; A2, 7.5 ± 3.4 [3]; and GA-1, 4.8 ± 2.6 [3]). Similarly, no differences in the extracellular content of catalase were observed (mean units per milligram of protein plus or minus the standard deviation from the mean for the number of experiments shown in brackets: V, 3.3 ± 3.0 [6]; A, 2.8 ± 2.3 [4]; A2, 1.8 ± 1.3 [4]; and GA-1, 2.4 ± 1.2 [4]).

Differences in intracellular catalase activity were evident, however; isolate A had two to four times the activity of the other isolates tested (mean units per milligram of protein plus or minus the standard deviation for the number of experiments shown in brackets: A, 645.0 ± 101.1 [2]; A2, 301.8 ± 125.0 [3], $P < 0.05$ versus isolate A; V, 256.0 ± 104.9 [4], $P < 0.02$ versus isolate A; and GA-1, 176.4 ± 33.8 [2], $P < 0.025$ versus isolate A). Intracellular catalase activity did not correlate with isolate virulence, as might be predicted. However, the high intracellular catalase activity of isolate A suggests an explanation for the observed resistance of this isolate to in vitro killing by PMNs despite its significant capacity to induce O_2^- production by PMNs in vitro.

Lack of O_2^- production and in vivo killing of *B. dermatitidis* isolates by PBMC. A comparison of *B. dermatitidis* isolates demonstrated no differences in susceptibility to in vitro killing by PBMC. The percentages of *B. dermatitidis* isolates killed by PBMC relative to medium alone were as follows (mean plus or minus the standard deviation from the mean for the number of experiments shown in brackets): V, 6.4 ± 7.9 [11]; VV, 6.2 ± 6.2 [2]; V40, 6.6 ± 5.1 [5]; A, 6.8 ± 7.9 [6]; A2, 6.7 ± 2.0 [2]; KL-1, 5.2 ± 4.9 [6]; and GA-1, 6.3 ± 3.7 [2]. *P* was >0.05 between isolates and for each isolate killed by PBMC relative to medium alone.

No detectable O_2^- production was observed in four separate experiments in which opsonized *B. dermatitidis* V, V40, A2, KL-1, or GA-1 was presented to PBMC in the same assay system as that used to detect O_2^- production by PMNs.

DISCUSSION

We report here the inverse correlation of in vivo virulence with in vitro killing by PMNs and the induction of PMN superoxide anion production by isolates of *B. dermatitidis*. While fungal strain-dependent differences in virulence for *B. dermatitidis* isolates have been reported (4, 5, 31), it has been suggested that fungal virulence is dependent on biochemical differences between fungal isolates (4, 5). To our knowledge, this is the first report to inversely correlate in vivo virulence of fungi with both in vitro susceptibility of fungi to killing by PMNs and fungal capacity to induce the production of host cell oxidative metabolites.

The descending order of in vivo virulence for the *B. dermatitidis* isolates tested was as follows: VV, V, V40, A, KL-1, A2, and GA-1. These findings are consistent with those reported for in vivo experiments with the conidial rather than the yeast form of the fungus: both the yeast and conidial forms of isolate V were highly virulent by the intranasal route and caused pathologic changes in the lungs of BALB/c mice; in contrast, neither the yeast nor the conidial form of avirulent isolate GA-1 caused disease (38).

Previous temporal studies (4, 5) of *B. dermatitidis* infection indicated that the events determining differences in virulence occur in the first few days of infection, before

B-cell- or T-cell-mediated immune responses can be mounted. The outcome of local encounters between PMNs and *B. dermatitidis* could fit the description of such a determining effect.

High-virulence isolate V was less susceptible to in vitro killing by PMNs from two sources (peripheral blood or peritoneal cavity) than low-virulence isolates A2, KL-1, and GA-1. It has been reported (37) that the percentage and number of PMNs in the lungs of mice infected with virulent isolate V continue to rise throughout infection until the mice die; in contrast, in mice infected with avirulent isolate GA-1, the influx of neutrophils subsides by the seventh day post-infection. These authors suggest that PMNs in vivo play a limited role in containing the virulent isolate, whereas they are effective in clearing the avirulent isolate (37). These results are consistent with ours in that more virulent isolates were less susceptible to killing by PMNs than avirulent isolates. However, in our study, even the virulent isolate was killed by PB-PMNs (38%), suggesting a role for PMNs in the containment of disease.

The extent of killing of the virulent isolates by PMNs is even greater with PMNs from mice previously immunized with *B. dermatitidis* cells; the percentage of the virulent isolate (V) killed by PMNs from immune mice was 51.7% of the fungal inoculum. Therefore, while the role for PMNs in fungal killing is more evident for avirulent isolates, PMNs may still play a significant role in defense against virulent isolates. This possibility is particularly evident when anamnestic responses of lymphoid cells are participating (28, 29). Frey et al. (19) reported that athymic nude mice were more susceptible than euthymic mice to intranasal challenge with either the yeast or the conidial form of *B. dermatitidis*. It has also been demonstrated that PMNs can be activated by supernatants of activated spleen cells (9) or by gamma interferon for enhanced fungal killing (27, 29). Therefore, PMNs may contribute further to the containment of fungal disease upon exposure to activating substances produced by the host in response to infection.

The role of PMNs may be especially important in the defense against blastomycosis, since it was reported that changes in macrophage number in mice infected with either a virulent or an avirulent isolate were minor despite the significantly different mortalities attributed to these isolates (37). In the present study, PBMC did not kill any *B. dermatitidis* isolate or produce O_2^- when assayed under the same conditions as those used for PMNs. These results do not rule out the possibility that under conditions of activation or maturation PBMC may become fungicidal. Pulmonary macrophages, unable to kill a virulent isolate of *B. dermatitidis*, were shown to become fungicidal after activation with gamma interferon or supernatants from activated lymph node cell cultures (10). No correlation was observed, however, between the in vivo virulence of an isolate and the capacity of activated pulmonary macrophages to kill the virulent (V) or attenuated (A, KL-1, and GA-1) isolates in vitro (10).

One possible mechanism for the observed differences in isolate virulence in vivo lies in the variable capacities of the isolates to induce superoxide anion production by PMNs. We demonstrated that high-virulence isolates V and V40 were poor inducers of O_2^- production in vitro. Low-virulence isolates produced quantities of O_2^- similar to those reported for human leukocytes ingesting latex particles (1). Low-virulence isolates A2, KL-1, and GA-1, however, stimulated 6 to 22 times more O_2^- production than did high-virulence isolates. These data suggest that it is the host's response (O_2^- production) to a property of the organism (ability to induce O_2^- production) which determines the virulence of a fungal isolate.

It has been reported that *B. dermatitidis* yeast cells can elicit a significant respiratory burst from human PMNs in vitro (33, 34). However, since our work demonstrates that results are dependent upon the isolate examined, one would need to know the virulence of the isolates tested before any comparisons with the results of the present study could be made. This would be the case even if ostensibly similar stocks of isolates were used (17), since storage and passage of isolates affect their virulence, as this study and others (4, 5) have indicated.

We report a unique finding regarding isolate A in that although it was intermediate in virulence in vivo and capable of inducing significant O_2^- production by PMNs in vitro, it was no more susceptible to in vitro killing by PMNs than were the virulent isolates. Isolate A, however, contained two to four times the intracellular catalase activity as did the other isolates tested, suggesting a possible mechanism for its enhanced resistance to in vitro killing by PMNs. Increased intracellular catalase content has been correlated with reduced in vitro susceptibility of fungi to killing by phagocytic cells (32). Other factors which differentiate isolate A from its parent, isolate V, include a markedly high content of fatty acids, an increased susceptibility to bile, the capacity to hydrolyze esculin, and an increased resistance to crystal violet in vitro (5). Our data suggest that isolate A may be more easily killed in vivo than the more virulent isolates because of host defense mechanisms other than those of PMNs.

Exogenously added catalase or SOD or both reduces PMN-mediated killing of *B. dermatitidis* yeast cells in vitro (30). No difference in extracellular catalase production between isolates was observed, and no extracellular SOD production by isolates was detected. The absence of detectable extracellular SOD does not exclude the possibility that differences in SOD production occur between isolates but that, because of the dilution effect of the medium, small quantities of this enzyme cannot be measured.

We conclude, therefore, that in vivo virulence inversely correlates with the in vitro susceptibility of fungal isolates to in vitro killing by PMNs and with their capacity to induce O_2^- production by PMNs in vitro. Isolate A demonstrates resistance to killing by PMN-generated oxygen metabolites which may result, at least in part, from its increased intracellular catalase content.

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