

Synergy of Itraconazole with Macrophages in Killing *Blastomyces dermatitidis*

ELMER BRUMMER,^{1,2,3*} PURUSHOTHAMA R. BHAGAVATHULA,²
LINDA H. HANSON,² AND DAVID A. STEVENS^{1,2,3}

Division of Infectious Diseases, Department of Medicine, Santa Clara Valley Medical Center,
751 South Bascom Avenue,^{1*} and California Institute for Medical Research,² San Jose,
California 95128-2699, and Stanford University Medical School, Stanford, California 94305³

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We examined *in vitro* interaction between the azole antifungal agents itraconazole and ketoconazole and macrophages and their activities against *Blastomyces dermatitidis*. Fungistatic and fungicidal concentrations for *B. dermatitidis* *in vitro* were assessed in a microculture system in which fungistasis was measured as inhibition of multiplication and fungicidal activity was measured as reduction of inoculum CFU. Resident peritoneal murine macrophages, which surround but do not phagocytize the fungus, were not fungicidal for *B. dermatitidis* isolates but were fungistatic for some isolates studied. Synergy was demonstrated when fungistatic concentrations (e.g., 0.01 µg/ml) of itraconazole, which limited growth 55% compared with that of controls, were cocultured with macrophages; this resulted in fungicidal activity (85% killing) against *B. dermatitidis* (ATCC 26199) in 72-h assays. This synergy could occur even if itraconazole was added after the macrophages had surrounded the fungus. Ketoconazole at fungistatic concentrations did not act synergistically with macrophages to kill *B. dermatitidis*. Lymph node lymphocytes could not substitute for macrophages in synergy with itraconazole to kill *B. dermatitidis*. When *B. dermatitidis* was separated by a filter from macrophages in Transwell cultures, fungicidal synergy with itraconazole was less efficient. Pretreatment of *B. dermatitidis* with itraconazole for 24 h did not render the fungus susceptible to killing by macrophages in the absence of itraconazole, whereas pretreatment of nonfungistatic macrophages with itraconazole rendered them fungistatic in a dose-dependent manner. Three other isolates were killed by otherwise fungistatic concentrations of itraconazole when the isolates were cocultured with macrophages. These findings indicate that one basis for the efficacy of itraconazole versus ketoconazole in treating blastomycosis could be synergy of a fungistatic concentration of itraconazole with macrophages in killing of *B. dermatitidis*.

As the outcome of deep fungal infection may be dependent on both host defenses and antifungal chemotherapy, the effect of antifungal drugs on immune responses is of interest. The immunomodulatory effect of amphotericin B, an antifungal agent in clinical use for 30 years, has been extensively studied. Less information is available concerning the interaction of the newer antifungal agents, the oral azoles, with host immune responses (1, 3, 7, 8, 10, 14, 16-19, 21, 22). We became interested in the possible effects of itraconazole on cellular immunity because of this triazole's efficacy against experimental murine blastomycosis (2) and human blastomycosis (4, 11, 20) and indications of superior *in vivo* efficacy of itraconazole compared with ketoconazole (2, 4) against blastomycosis, which was not predicted from either inhibition or killing of *B. dermatitidis* (2) by these drugs *in vitro*.

MATERIALS AND METHODS

Mice. Male BALB/cByJMR mice, 8 to 12 weeks of age, were obtained from the specific-pathogen-free breeding colony of the California Institute for Medical Research, San Jose.

Reagents and media. Tissue culture medium RPMI 1640 with L-glutamine, heat-inactivated fetal bovine serum, and penicillin-streptomycin (10,000 U/ml and 10,000 µg/ml, respectively) were purchased from GIBCO Laboratories (Grand Island, N.Y.). Complete tissue culture medium

(CTCM) consisted of 79 ml of RPMI 1640, 10 ml of fetal bovine serum, 10 ml of fresh mouse serum, and 1 ml of penicillin-streptomycin. Ketoconazole and itraconazole were supplied as powders by Janssen Research and Development, New Brunswick, N.J. Itraconazole (10 mg) was dissolved in 1 ml of a solution consisting of 50% acetone and 50% 0.2 N HCl. This stock solution was stored in the dark at room temperature. Ketoconazole (50 mg) was dissolved in 0.2 N HCl and then diluted in distilled water to 2 mg/ml. Portions of this stock preparation were stored at -20°C.

Fungi. The isolates of *Blastomyces dermatitidis* used in this study were shown to be virulent in a murine model of pulmonary blastomycosis (12) or systemic infection (15). Most of the experiments used isolate ATCC 26199. Other isolates tested were ATCC 60636, SC 599, and Har. Inocula of *B. dermatitidis* were prepared from cultures grown for 72 h on blood agar plates at 35°C. The yeast cells were washed twice in 4 ml of saline. Fungal units (single cells or multicellular aggregates) were counted with a hemacytometer and suspended in CTCM at 5,000 U/ml. Viable CFU of inocula were determined by plating 1 ml of an appropriate dilution in quadruplicate on blood agar plates.

Peritoneal macrophages. Resident peritoneal cells were collected from the abdominal cavity by repeated lavage with a total of 10 ml of RPMI 1640 containing heparin at 10 U/ml per mouse. After centrifugation (200 × g, 10 min), pelleted cells from five mice were pooled in 10 ml of RPMI 1640. Pooled cells were centrifuged, and the pelleted cells were suspended in 1 ml of CTCM not containing fresh mouse

* Corresponding author.

serum. Peritoneal cells were counted with a hemacytometer, and their concentration was adjusted in the same medium to 5×10^6 /ml. Peritoneal cell suspensions (0.1 ml per flat-bottom well of an A/2 microtest plate [Costar, Cambridge, Mass.]) were dispensed. After 2 h at 37°C in 5% CO₂-95% air, nonadherent cells were removed by aspiration and one wash with fresh medium. Adherent cell monolayers, which were 90% macrophages, consisted of approximately 2.5×10^5 cells per well (6). For experiments in Transwell chambers (Costar), 0.5 ml of 4×10^6 peritoneal cells per ml of CTCM (without fresh mouse serum) was dispensed per flat-bottom well (15-mm-diameter lower chamber) and incubated for 2 h at 37°C in 5% CO₂-95% air. Nonadherent cells were removed by aspiration and one rinse with medium. Monolayers consisted of approximately 2×10^6 adherent peritoneal cells per well.

Cocultures. Macrophage monolayers were challenged with *B. dermatitidis* isolates (0.1 ml of 5,000 fungal units per ml of CTCM) in the absence or presence of various concentrations of antifungal agents. In three representative experiments, the number of CFU in these counted inocula was 443 ± 118 (mean \pm standard deviation), a plating efficiency of 88.6%. Control cultures consisted of *B. dermatitidis* alone in the absence or presence of various concentrations of ketoconazole or itraconazole. Other controls contained 2.5×10^5 nonadherent lymph node cells (instead of macrophages) per well plus *B. dermatitidis* in the absence or presence of antifungal agents. Microscopic observation of the cocultures has shown that the macrophages surround and cover much of the fungal surface but do not appear to successfully internalize (phagocytize) the yeast cells (6).

Transwell chambers. A Transwell apparatus has two vertical chambers bathed in the same menstrium and separated by a 0.4- μ m-pore-size membrane. Macrophage monolayers in the lower chamber were coincubated with 2,000 fungal units of *B. dermatitidis* in the upper (6.5-mm-diameter) chamber. The total volume of CTCM in the Transwell apparatus was 0.8 ml. Transwell cocultures were incubated in the absence or presence of various concentrations of itraconazole. Control Transwell cultures consisted of *B. dermatitidis* in the upper chamber without macrophages below in the absence or presence of itraconazole. Concurrent contact cocultures in a Transwell apparatus were established, with *B. dermatitidis* being added directly to the macrophage monolayer and incubated in the absence or presence of itraconazole. Controls for these contact cocultures contained *B. dermatitidis* alone with or without itraconazole.

Measurement of fungicidal and fungistatic activities. Quadruplicate cocultures were incubated for 72 h (unless otherwise specified) at 37°C in 5% CO₂-95% air and then harvested with distilled water. This treatment removed cells from the well and lysed macrophages as assessed by microscopic examination. Microscopy also revealed that the distribution of fungal cells per fungal unit in control and experimental materials was not different. The number of CFU per well was determined by plating 1 ml of the diluted well contents (final volume, 10 ml) on a blood agar plate and counting the number of colonies after 4 days at 35°C. Duplicate Transwell cultures were harvested by removing the CTCM from the lower chamber and harvesting the *B. dermatitidis* from the upper chamber with distilled water. The number of CFU per chamber was determined by plating an appropriate volume of harvested material on blood agar plates. Contact cocultures in Transwell wells were har-

vested, and the number of CFU per well was determined as described above for cocultures in MicroTest plate wells.

In three representative experiments, the CFU increased a mean of 20.3-fold over 72 h in CTCM alone. Activities of drug, macrophages, and combinations of both and other experimental conditions were always compared with growth in concurrent control (CTCM) cultures in each experiment, and the data were expressed as a percentage relative to results with that control. Fungicidal activity was measured as reduction of inoculum CFU: percent killing = $[1 - (\text{CFU in experimental culture}/\text{CFU in inoculum})] \times 100$. Fungistatic activity was measured as inhibition of multiplication: percent inhibition = $[1 - (\text{CFU in experimental culture}/\text{CFU in control culture})] \times 100$.

Lysate and supernatant tests. Macrophages in MicroTest plate wells were cultured in CTCM at 37°C in 5% CO₂-95% air for 72 h in the presence or absence of various concentrations of itraconazole. Culture supernatants were aspirated and pooled. The fungicidal or fungistatic activities of undiluted supernatants were tested by adding the supernatants to *B. dermatitidis* (5,000 fungal units per ml) and dispensing 0.1 ml per well of a MicroTest plate. Control supernatants consisted of CTCM alone incubated for 72 h with or without itraconazole.

Macrophages were lysed by addition of 0.02 ml of distilled water per well followed by three cycles of freezing (-70°C) and thawing. Lysates from several wells were pooled for testing. CTCM was added to lysates (0.08 ml of CTCM per 0.02 ml of lysate), and the fungicidal or fungistatic activity was assessed by adding *B. dermatitidis* (5,000 fungal units per ml) to the lysate and then dispensing 0.1 ml per well of a MicroTest plate.

Supernatant and lysate cultures were incubated for 72 h at 37°C in 5% CO₂-95% air, and the number of CFU per culture was determined as described above.

Pretreatment of *B. dermatitidis* with itraconazole. To investigate the possible effects of itraconazole interaction or concentration of drug by *B. dermatitidis*, the fungus was pretreated for 24 h. For pretreatment, *B. dermatitidis* was incubated with fungicidal (71% killing) concentrations of itraconazole (0.1 μ g/ml) for 24 h, and then the drug was removed. Treated *B. dermatitidis* was cocultured with macrophages for 48 h without itraconazole or with fungicidal or fungistatic concentrations of drug. Cultures were harvested, and the number of CFU per culture was determined as described above.

Statistics. Comparisons between groups were analyzed by Student's *t* test, with significance assumed to be $P < 0.05$.

RESULTS

Synergy of itraconazole with macrophages. In studies at 72 h of coculture, 0.1 μ g of itraconazole per ml was fungicidal (92% \pm 12%) to *B. dermatitidis* (ATCC 26199), and 0.02 and 0.01 μ g/ml were not (Fig. 1). However, the lower concentrations of itraconazole were fungistatic for *B. dermatitidis*, e.g., 56% \pm 19% and 45% \pm 14% ($n = 3$ experiments) at 0.02 and 0.01 μ g/ml, respectively. Macrophages alone were not fungicidal or fungistatic for this isolate in these experiments. In subsequent experiments with this isolate, macrophages alone were never fungicidal and variably showed no fungistatic ability or only a slight fungistatic effect (up to 24%).

In cocultures of itraconazole and macrophages, fungistatic concentrations of itraconazole (0.01 and 0.02 μ g/ml) acted synergistically with macrophages to kill *B. dermatitidis* (85% \pm 1% and 91% \pm 6%, respectively) (Fig. 1).

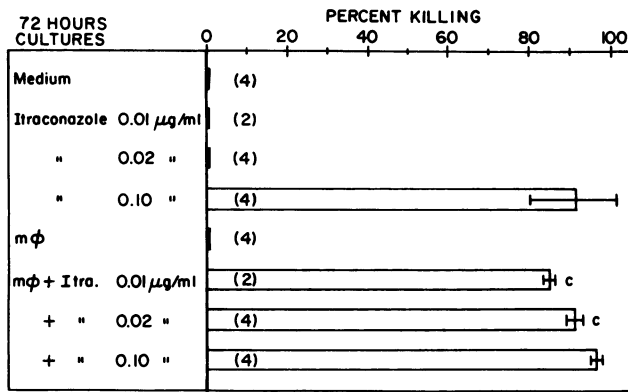


FIG. 1. Synergy between itraconazole and macrophages (mφ) in killing of *B. dermatitidis* ATCC 26199. Shown are percent reduction of inoculum CFU, number of experiments (in parentheses), and significant killing ($P < 0.001$) when fungistatic itraconazole alone is compared with itraconazole (Itra.) plus macrophages (c).

In 24-h cocultures, *B. dermatitidis* was significantly killed by itraconazole at higher concentrations (0.1 and 0.4 µg/ml) but not at lower concentrations (0.02 µg/ml) (two or three experiments; data not shown). In 24-h cocultures of macrophages and itraconazole, killing of *B. dermatitidis* was enhanced compared with drug alone in the presence of a low concentration (0.02 µg/ml); however, this difference was not statistically significant.

Lack of synergy between ketoconazole and macrophages. Ketoconazole was fungicidal for *B. dermatitidis* (ATCC 26199) at 0.1 µg/ml (100%) and was only fungistatic (51% ± 15%; $n = 2$) at 0.01 µg/ml in 72-h cultures. In contrast to itraconazole, ketoconazole at a fungistatic concentration did not act synergistically with macrophages to kill *B. dermatitidis*. Fungistatic activity of ketoconazole (0.01 µg/ml) in coculture with macrophages was increased to 64% ± 1% ($n = 2$), but this increase was not significant ($P > 0.05$).

A third, dose-response experiment was performed to ascertain whether some intermediate concentration of ketoconazole alone would be fungistatic but act synergistically with macrophages to become fungicidal. The aggregated data of the ketoconazole experiments are shown in Fig. 2. In the third experiment, 0.02 µg of ketoconazole per ml was also fungistatic (53%) but did not become fungicidal with macrophages. In addition, 0.04 µg of ketoconazole per ml was tested in this experiment (data not shown) and was fungicidal (67%), though to a lesser extent than the higher concentrations tested, but its activity was not significantly enhanced by macrophages.

Lack of synergy between itraconazole and lymphocytes. Lymph node cells depleted of macrophages by adherence to plastic were tested for their abilities to act synergistically with itraconazole to kill *B. dermatitidis* (ATCC 26199). Fungistatic concentrations of itraconazole (0.01 and 0.02 µg/ml) in the presence of lymphocytes were not fungicidal, nor was fungistatic activity significantly increased. By contrast, these concentrations of itraconazole had acted synergistically with macrophages to kill *B. dermatitidis* in 72-h cultures, as described above. On the other hand, lymph node cells not depleted of macrophages did act synergistically with itraconazole (0.02 µg/ml) to produce fungicidal activity against *B. dermatitidis* (73% killing). These findings suggest that macrophages or adherent lymph node cells but not

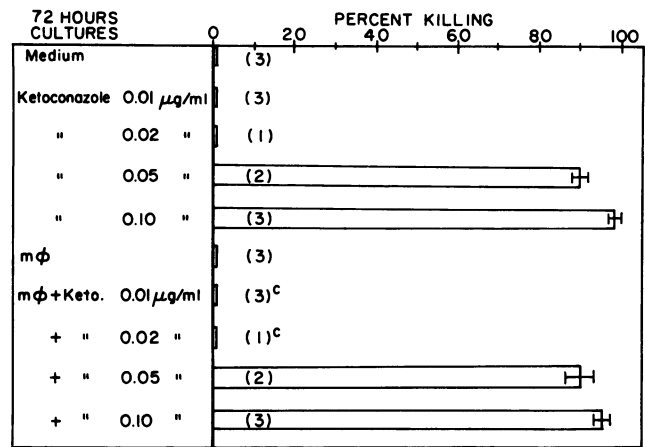


FIG. 2. Lack of synergy between ketoconazole and macrophages (mφ) in killing of *B. dermatitidis* ATCC 26199. Shown are percent reduction of inoculum CFU and number of experiments (in parentheses). There was no significant killing ($P > 0.05$) by macrophages plus fungistatic ketoconazole (Keto.) versus fungistatic ketoconazole alone (c).

nonadherent lymphocytes can act synergistically with itraconazole to kill *B. dermatitidis*.

Contact requirement for optimal macrophage and itraconazole synergy. To investigate the effect of macrophage contact with *B. dermatitidis* in the production of synergy with itraconazole in fungicidal activity, Transwell cultures were used. Itraconazole alone was fungistatic at 0.01 and 0.02 µg/ml in these wells (87 and 93%) at 72 h and was fungicidal at 0.1 µg/ml (47% ± 1%).

In contrast, when macrophages were present, there were some important differences in the results depending on whether the macrophages and fungi were permitted direct contact or were separated by the membrane. When the fungus was separated from the macrophage monolayer, the

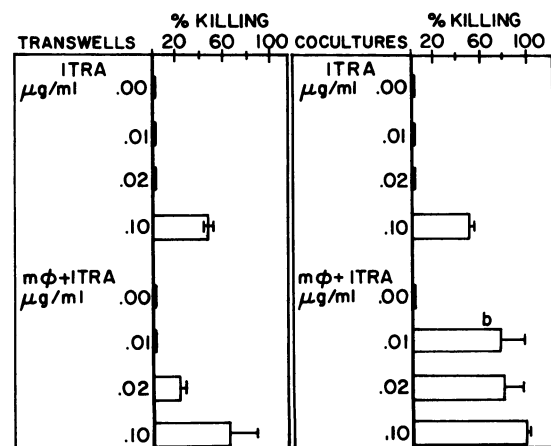


FIG. 3. Effect of contact on synergy between itraconazole (ITRA) and macrophages (mφ) in killing of *B. dermatitidis*. Shown are percent reduction of inoculum CFU (mean ± standard deviation; $n = 2$) and significant killing ($P < 0.001$) in contact cultures (cocultures) of macrophages and fungistatic itraconazole (0.01 µg/ml) (b). The latter contrasts with lack of killing in Transwell cultures of itraconazole (0.01 µg/ml) and macrophages with Transwell membrane present.

TABLE 1. Effect of pretreatment of *B. dermatitidis* with itraconazole

Amt ($\mu\text{g/ml}$) of itraconazole used for pretreatment	CFU \pm SD ^a				
	24 h	Posttreatment (48 h)			
		CTCM	M ϕ	M ϕ + itraconazole	Itraconazole
0.0	1,230 \pm 96	10,000 \pm 500	9,875 \pm 1,280	9,750 \pm 530	10,060 \pm 500
0.01	600 \pm 51	4,700 \pm 1,370	3,600 \pm 260	70 \pm 34 ^b	1,985 \pm 320
0.02	613 \pm 66	4,910 \pm 730	3,785 \pm 350	80 \pm 28 ^b	820 \pm 72
0.1	172 \pm 61 ^b	1,400 \pm 8	1,314 \pm 300	22 \pm 17 ^b	120 \pm 26 ^b

^a Data are results from quadruplicate cultures. CFU \pm standard deviation at 0 h was 447 \pm 77. M ϕ , macrophages. Values for each concentration in CTCM and M ϕ columns are not significantly different.

^b Fungicidal, i.e., reduction of 0-h CFU.

fungistatic itraconazole concentration of 0.01 $\mu\text{g/ml}$ did not become fungicidal (Fig. 3, left). In concurrent contact cocultures, this concentration of drug acted synergistically with macrophages to kill the fungi (75% \pm 26%) (Fig. 3, right). Compared with the activity of the same combination with the membrane present, the difference was significant ($P < 0.001$).

However, when a drug concentration of 0.02 $\mu\text{g/ml}$ (fungistatic without macrophages) was present in cultures with macrophages and with the membrane present, there was significant ($P < 0.01$) killing (24% \pm 2%) of fungi (Fig. 3, left) despite lack of macrophage-fungus contact. The killing by this combination was greater (80% \pm 19%) when contact was permitted in cocultures (Fig. 3, right). Moreover, in this large-well culture system, killing by a fungicidal concentration of itraconazole (0.1 $\mu\text{g/ml}$) was slightly greater with macrophages and the membrane present (47 to >60%; Fig. 3, left) but was significantly ($P < 0.001$) increased in cocultures (99% \pm 1%; Fig. 3, right).

These results indicate a requirement for macrophage-fungus contact for optimal synergy with itraconazole in killing *B. dermatitidis*.

Effects of itraconazole pretreatment of *B. dermatitidis*, addition of itraconazole after macrophage-fungus contact, or pretreatment of macrophages. Incubation of *B. dermatitidis* with fungicidal (74% killing) concentrations of itraconazole (0.1 $\mu\text{g/ml}$) for 24 h and then washes to remove drug did not render the fungus more susceptible to macrophages in the absence of itraconazole in a 48-h assay (Table 1, CTCM versus M ϕ columns). In contrast, macrophages in the continuing presence of fungistatic concentrations of itraconazole (0.01 and 0.02 $\mu\text{g/ml}$) were fungicidal (85% killing) for pretreated *B. dermatitidis* (Table 1, M ϕ + itraconazole column). These findings suggest that 24-h exposure of *B. dermatitidis* to fungicidal concentrations of itraconazole does not render the fungus susceptible to killing by macrophages and that continuous presence of the drug is required for optimal fungicidal activity.

One experiment examined whether itraconazole could still act synergistically with macrophages if drug and phagocyte were not added simultaneously but if macrophages were allowed to surround the fungi first (6). In this experiment, itraconazole was added to macrophage cultures 2 h after macrophages and *B. dermatitidis* had been in contact. A concentration of itraconazole (0.02 $\mu\text{g/ml}$) which was fungistatic (41% in this experiment) without macrophages acted synergistically with macrophages, even if added after macrophage-fungus contact, producing 92% killing at 72 h. Fungicidal concentrations of itraconazole alone (0.1 or 0.4 $\mu\text{g/ml}$) added to macrophages after they had surrounded *B. dermatitidis* cells were also fungicidal in this system.

Another experiment studied the effect of 24-h pretreatment of macrophages with concentrations of itraconazole which had been shown to be fungicidal (0.1 $\mu\text{g/ml}$) or fungistatic (0.01 and 0.02 $\mu\text{g/ml}$). Macrophages were then washed and cocultured with fungi for 48 h. In this study, the macrophages which were not fungistatic without treatment became significantly ($P < 0.05$) fungistatic in a dose-dependent manner (38, 56, and 86% inhibition of multiplication by pretreatment with 0.01, 0.02, and 0.1 $\mu\text{g/ml}$, respectively) after pretreatment with all three concentrations.

Activity of culture supernatants or macrophage lysates. To further study the possibility that macrophages act synergistically with fungistatic concentrations of itraconazole by metabolizing the drug to a more potent form or by cellular concentration of the drug by macrophages, the following experiments were done. Macrophages were cultured with various concentrations of itraconazole for 72 h, and the supernatants were then harvested and tested against *B. dermatitidis*. Macrophages from these experiments were lysed, and lysates were tested for fungicidal or fungistatic activity.

We found that coculture supernatants with fungicidal concentrations of itraconazole (0.08 and 0.04 $\mu\text{g/ml}$) remained fungicidal (98.5% \pm 7% and 73.5% \pm 0.7% killing, respectively). This activity was not different from the activity of the corresponding concentration of itraconazole in CTCM. Supernatants from macrophage cultures which had contained fungistatic concentrations of itraconazole (0.01 and 0.02 $\mu\text{g/ml}$) did not become fungicidal for *B. dermatitidis*. Moreover, lysates of macrophages from cocultures with fungicidal or fungistatic concentrations of itraconazole did not show either activity against *B. dermatitidis*.

Other *B. dermatitidis* isolates. To determine whether macrophages and itraconazole act synergistically against other isolates of *B. dermatitidis*, three other isolates (Har, SC 599, and ATCC 60636) were tested in this system. The fungicidal concentration of itraconazole for these isolates was higher (0.5 $\mu\text{g/ml}$) than that for ATCC 26199 (0.1 $\mu\text{g/ml}$) (Table 2). These isolates, unlike ATCC 26199, which frequently escapes from macrophage inhibition of multiplication in 72 h (6; present study), had their multiplication inhibited by macrophages compared with that in CTCM; i.e., multiplication was inhibited 14% (ATCC 60636), 77% (Har), or 78% (SC 599) (data not shown).

Macrophages were able to act synergistically with concentrations of itraconazole (0.1 $\mu\text{g/ml}$) that were fungistatic alone against all three of these isolates (Table 2). With the Har isolate, 0.1 μg of itraconazole per ml was fungicidal (68% \pm 28%) in the presence of macrophages. Likewise, this fungistatic concentration of itraconazole acted synergistically with macrophages to kill SC 599 (60%) and ATCC

TABLE 2. Synergy of macrophages and itraconazole in killing of *B. dermatitidis* isolates

Treatment ($\mu\text{g/ml}$)	% Reduction (mean \pm SD) in ^a :			
	ATCC 26199	Har	SC 599	ATCC 60636
Itraconazole				
1.00		100	100	80
0.50		86	97	86
0.10	93 \pm 12	0 \pm 0 ^b	0 \pm 0 ^b	0 \pm 0 ^b
0.02	0 \pm 0 ^b	0 \pm 0	0 \pm 0	0 \pm 0
0.01	0 \pm 0 ^b	0 \pm 0	0 \pm 0	0 \pm 0
Itraconazole + Mϕ				
1.00		100		
0.50		100		
0.10	97 \pm 2	68 \pm 28 ^c	60 ^c	49 ^c
0.02	91 \pm 6 ^c	0 \pm 0	36 ^c	35 ^c
0.01	85 \pm 1 ^c	0 \pm 0	34 ^c	40 ^c

^a $n = 2$ experiments. Results are reduction of inoculum CFU in 72 h. A single value (with no standard deviation) is the mean percent reduction of inoculum CFU in quadruplicate cultures in a single experiment.

^b Fungistatic (data not shown).

^c Synergy ($P < 0.05$, comparing results for drug plus M ϕ with results for drug alone).

60636 (49%) (Table 2), and even lower (nonfungistatic) concentrations of itraconazole did so with SC 599 and ATCC 60636 (Table 2).

These results show that susceptibility of *B. dermatitidis* isolates to itraconazole may vary; nevertheless, at fungistatic concentrations of itraconazole, macrophages can act synergistically with the drug for killing of the isolates.

DISCUSSION

We report here that fungistatic concentrations of itraconazole but not ketoconazole act synergistically with resident murine peritoneal macrophages to kill *B. dermatitidis*. This novel finding, in addition to the perhaps more obvious possible differences in pharmacokinetics and/or tissue penetration, may be one basis for the enhanced efficacy of itraconazole over ketoconazole in treating murine blastomycosis (2) and human blastomycosis (4).

Although this is the only study that we are aware of that demonstrated synergy of itraconazole with phagocytes in killing a thermally dimorphic fungal pathogen, others reported synergy (1, 17), an additive effect (22), or no effect (14) in studies with other fungal pathogens. Previous studies with ketoconazole have reported that it increases some monocyte antimicrobial functions and depresses others (7), that it depresses (19) or has no effect (21) on neutrophil antimicrobial function, that it acts synergistically with (1, 3, 8) or antagonizes (19) neutrophil antifungal activity or has no effect (14, 16, 18), or that there is synergy only if the fungus is pretreated with the drug (10).

We found that contact between *B. dermatitidis* and macrophages was necessary for optimal synergy with itraconazole in killing and that synergy was demonstrated even if itraconazole was added after *B. dermatitidis* was surrounded by macrophages. This observation indicates that macrophages do not constitute a barrier that might protect *B. dermatitidis* from the action of itraconazole.

The mechanism by which fungistatic or nonfungistatic macrophages and fungistatic concentrations of itraconazole interact to produce a fungicidal effect is not known. Since itraconazole binds to a variety of mammalian cells (13), it is possible that the macrophages concentrate the drug from the

surrounding milieu and deliver it in an efficient way. In studies with dead macrophages, Perfect et al. (17) suggested that itraconazole binds to macrophages. It is possible that itraconazole activates macrophages, resulting in a cell with enhanced antifungal power. The studies of pretreatment of macrophages would support either or both of these explanations.

The studies of pretreatment of the fungus do not suggest that itraconazole sensitizes this fungus for an otherwise nonlethal encounter with macrophages. The possibility that mammalian cells deplete the medium of components required for fungal survival appears excluded by the experiments with lymphocytes, which also appear to exclude the possibility that metabolites of merely any host cell are hostile to the fungi in this system. A fifth possibility is that macrophages, specifically, generate a product that is capable of acting synergistically with itraconazole. Synergy was not optimal in the Transwell experiments in which physical contact of macrophage and fungus was prevented but did occur at the higher fungistatic itraconazole concentration tested, which would support the idea of a synergistic macrophage product. That synergy did not occur in the Transwell experiments at lower drug concentrations may suggest that direct contact is superior for delivery of the putative macrophage antifungal product or that a second antifungal mechanism could also ensue with contact. The putative macrophage antifungal product mentioned could be a cytokine, oxidative metabolite, or concentrated and rereleased drug. A sixth possibility, that macrophages convert itraconazole to a more potent form, seems unlikely from the experiments with macrophage supernatants. Finally, macrophages could, in effect, decrease the fungal inoculum by aggregating the fungi and segregating some of the fungal inoculum from the drug. Since itraconazole's in vitro activity is likely inoculum dependent like that of other azoles (5), an enhanced antifungal activity against a smaller inoculum would be expected. Both the Transwell synergism data and the data with ketoconazole (another inoculum-dependent antifungal agent in vitro) militate against this.

Since the diameter of a macrophage is estimated to be 15 to 25 μm (9), we thus estimate the total intracellular volume of macrophages in one well as 1 mm^3 (1% of the total liquid volume of the well). If 0.04 μg of itraconazole per ml were concentrated as much as 50-fold by the macrophages, the supernatant concentration would fall 50%, thus lowering the concentration of the drug to a nonfungicidal level. Since this did not occur in our experiments, we assume that if macrophages concentrate the drug, the concentration must be <50-fold. Moreover, lysate experiments suggest that if macrophages concentrate the drug, concentration must be <12.5-fold. A 12.5-fold concentration of 0.08 μg of itraconazole per ml, producing a macrophage cellular concentration of 1 $\mu\text{g/ml}$, would, after the dilution for assay that was needed, produce a concentration in the diluted lysate of 0.01 $\mu\text{g/ml}$, which is the limit of sensitivity of the antifungal assay used here. However, because of the dilution factor in our assays, we cannot exclude the possibility of a factor of concentration of <12.5-fold but sufficient to produce a fungicidal concentration in the macrophage. The Transwell data do militate to some extent against the argument that the mechanism relates to concentration of drug by macrophages.

Four isolates of *B. dermatitidis* which were virulent in mice were all susceptible to killing by itraconazole; however, the fungicidal concentration for one isolate (ATCC 26199) was fivefold lower than those for the other isolates,

and fungistatic concentrations were also lower. Fungicidal synergy was demonstrated with all isolates.

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