# Intracellular Activity of Voriconazole, Fluconazole, and Itraconazole Against *Candida albicans* in Human Monocytes With and Without Activation by GM-CSF and TNF-□

Aldona L. Baltch, MD*†
Raymond P. Smith, MD*†
William J. Ritz, MS*†
Lawrence H. Bopp, PHD*
Phyllis B. Michelsen, ScD*†

**Infectious Disease Section, Stratton Veterans Affairs Medical Center, Albany, New York*
*†Albany Medical College, Albany, New York*

**KEY WORDS:** voriconazole, intracellular activity, *Candida albicans*, azoles, cytokines

## ABSTRACT

**Background:** Disseminated infections caused by *Candida albicans* continue to be associated with high mortality. Monocytes are important host phagocytic cells which, when activated by cytokines, can have increased killing activity. This study compares the intracellular activity of three azoles (fluconazole, voriconazole, and itraconazole) in human monocyte-derived macrophages (MDM) activated or not activated by cytokines.

**Design and Methods:** Human MDM monolayers prepared from heparinized blood of healthy volunteers were infected with *C albicans* (fluconazole-resistant [$flu^R$] strains 8336-2 and ATCC 64550; fluconazole-susceptible [$flu^S$] strains T=6, ATCC 56882, and ATCC 90028). Antifungal agents (1 x minimal inhibitory concentration) were added following a 1-h phagocytosis time. Numbers of viable *C albicans* from MDM lysates were determined at 0, 24, and 48 h.

**Results:** For two of the five *C albicans* strains (ATCC 90028 and T=6), all azoles had similar activity at 24 h. There were slight differences at 48 h. For strains ATCC 64550 and ATCC 56882, the most effective azoles were fluconazole and itraconazole, respectively, and voriconazole was most effective against strain 8336-2. Voriconazole was least effective against strain ATCC 56882, and itraconazole was least effective

against strain ATCC 64550. Significant effects of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor  occurred only with voriconazole and were strain-dependent. Activity of voriconazole was enhanced, inhibited, or unaffected by these cytokines.

**Conclusions:** This study clearly indicates that in the presence of different azoles and cytokines, the intracellular anticandidal activity of human MDM cannot be predicted and may differ for individual *C albicans* strains.

## INTRODUCTION

Disseminated candidiasis continues to be a serious fungal infection, especially in debilitated and immunocompromised hosts.[1-3] Furthermore, fluconazole-resistant (flu$^R$) *Candida albicans* strains have been found in such patients with increased frequency.[2,3] Newer azoles, voriconazole and itraconazole, have increased activity against flu$^R$ *C albicans* as well as other *Candida* species.[4-9]

Azoles are also known to enter phagocytic cells.[10,11] Although antimicrobial agents are important in the treatment of infection caused by *Candida* spp., host factors, including phagocytic cells, are also important in patient survival.[12] Cytokine-activated phagocytic cells have been shown to have increased inhibitory or fungicidal activity, largely related to oxygen burst mechanisms.[13-19]

Furthermore, the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor  (TNF-) have been shown to enhance human monocyte anticandidal activity.[13-15,17-19] The addition of cytokines to appropriate antifungal agents in the therapy of immunocompromised patients with disseminated candidiasis and hepatosplenic candidiasis has been reported to increase patient survival.[20,21] In addition, TNF- has a protective role in a murine model of systemic candidiasis.[22]

Azoles inhibit or kill *C albicans* by inhibiting the cytochrome P-450–dependent enzyme 14- sterol demethylase, blocking the sterol biosynthesis pathway essential for the production of a functional membrane.[23-28] Newer azoles, voriconazole and itraconazole, are more active than the earlier azole, fluconazole, against *C albicans*.

The purpose of this study was to evaluate the intracellular antifungal activity of three azoles (fluconazole, itraconazole, and voriconazole) against flu$^R$ and fluconazole-susceptible (flu$^S$) *C albicans* strains in human monocyte-derived macrophages (MDM), and to determine if these activities differed in unactivated MDM and MDM activated with the cytokines GM-CSF and TNF-. Production of GM-CSF and TNF- by MDM exposed to voriconazole, fluconazole, and itraconazole was also evaluated.

## MATERIALS AND METHODS

### *Candida* Strains

*C albicans* strain T=6, originally isolated from the blood of a patient with candidemia, was provided by Wadsworth Laboratories, New York State Department of Health, Albany, NY. *C albicans* strain 8336-2 is a clinical isolate obtained from the throat of a patient with AIDS at the Stratton Veterans Affairs Medical Center, Albany, NY. Three other *C albicans* strains, ATCC 64550, ATCC 90028, and ATCC 56882, were obtained from the American Type Culture Collection, Manassas, VA.

### Antimicrobial Agents

Voriconazole and fluconazole were provided by Pfizer Laboratories (Sandwich, Kent, England). Itraconazole was purchased from Research Diagnostics Inc. (Flanders, NJ). Antibiotic solutions were made fresh for each experiment in accordance with the suppliers' instruc-

tions, filter-sterilized, and used immediately. Minimal inhibitory concentrations (MICs) for fluconazole, voriconazole, and itraconazole, respectively, determined according to the National Committee for Clinical Laboratory Standards method M27-A2,[29,30] were as follows: ATCC 64550: 32, 0.5, 0.25 µg/mL; 8336-2: 64, 2, 0.5 µg/L; T=6: 1, 0.06, 0.25 µg/mL; ATCC 90028: 0.5, 0.03, 0.125 µg/mL; and ATCC 56882: 0.25, 0.03, 0.03 µg/mL.

**Preparation of Human Monocytes**

Monocytes were prepared from heparinized blood of healthy human donors who had signed the informed consent form approved by the Institutional Review Board of the Albany Medical College and Stratton Veterans Affairs Medical Center, Albany, NY. Mononuclear cells were separated from whole blood by using Histopaque-1077 (Sigma Chemical Co., St. Louis, MO). The resulting mononuclear preparation was ≥ 98% pure. The separated cells were resuspended at a concentration of 2 x $10^6$ cells/mL in RPMI+ (RPMI 1640 medium [Sigma] containing 10% fetal bovine serum [Sigma, cat. # F-2442]). Cell viability, determined by using the trypan blue exclusion test, was ≤ 98%.

**Cytokines**

Cytokines and enzyme-linked immunosorbent assay (ELISA) kits for cytokine determinations were obtained from R & D Systems, Inc. (Minneapolis, MN). MDM were activated with GM-CSF and TNF-α at concentrations of 100 U/mL.

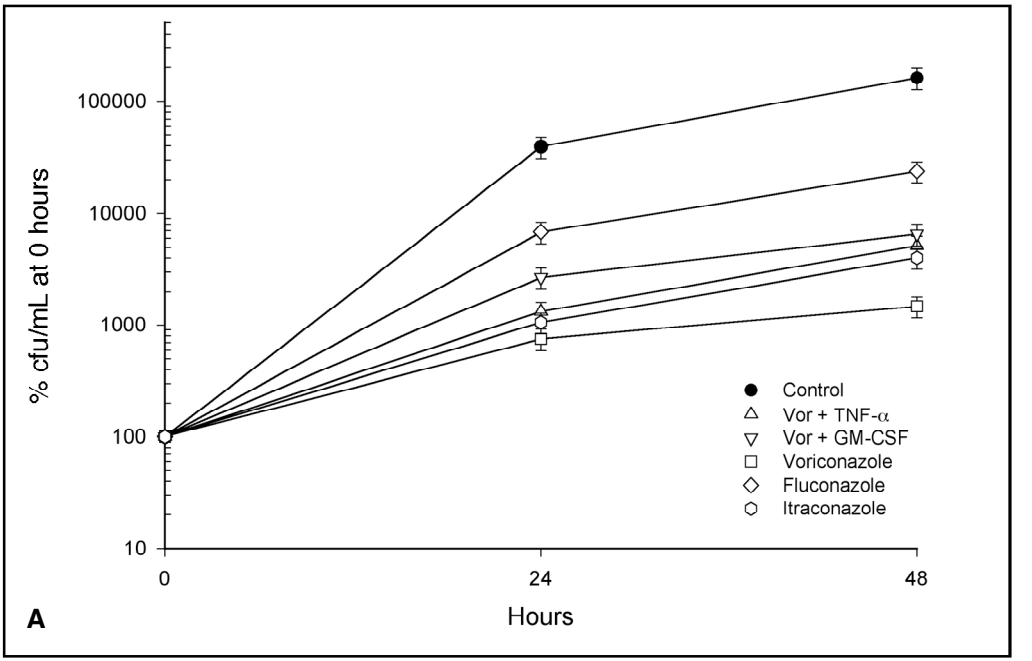
**Anticandidal Activities of Voriconazole, Fluconazole, and Itraconazole in MDM**

Human mononuclear cells (2 x $10^6$/mL) were delivered to the wells of 24-well plates (Corning/Costar Corp., Cambridge, MA) at a volume of 1 mL/well and allowed to adhere for 72 h. Medium and nonadherent cells, including lymphocytes, were aspirated from the wells. The adherent, confluent monocyte layers, from this point referred to as MDM, were washed once with RPMI+. RPMI+, with or without GM-CSF and TNF-α (100 U/mL), was added gently to the surfaces of monolayers in duplicate wells. Plates were incubated at 37˚C for 24 h in an atmosphere containing 5% $CO_2$. The medium was removed from the wells by aspiration and a 1-mL aliquot of *C albicans* containing 2 x $10^4$ cells suspended in RPMI+ was then added to each well. After allowing 1 h for phagocytosis, nonphagocytosed yeast were removed by aspiration and the monolayers were washed once with RPMI+. One mL of RPMI+, with or without cytokines as appropriate, was then added to each monolayer.

Antifungal drugs (1 x MIC for each particular *C albicans* strain) were then added to appropriate wells. Following incubation of the plates at 37˚C for 0, 24, or 48 h in an atmosphere containing 5% $CO_2$, the medium was removed, the MDM were lysed with sterile distilled water, and the number of viable yeast in the lysates was determined by using the standard plate count method (24 h incubation at 37˚C) and Sabouraud dextrose agar (Sigma).

**Effects of Voriconazole, Fluconazole, and Itraconazole on Cytokine Production by MDM**

MDM monolayers prepared as described above were studied for the production of GM-CSF and TNF-α following exposure to serum-attainable concentrations of voriconazole, fluconazole, and itraconazole (2.5, 8.0, and 0.4 µg/mL, respectively). Controls included *Escherichia coli* serotype 055:B5 lipopolysaccharide (LPS;1 µg/mL) and MDM monolayers alone. Cytokine concentrations in the culture media overlying the monolayers were determined



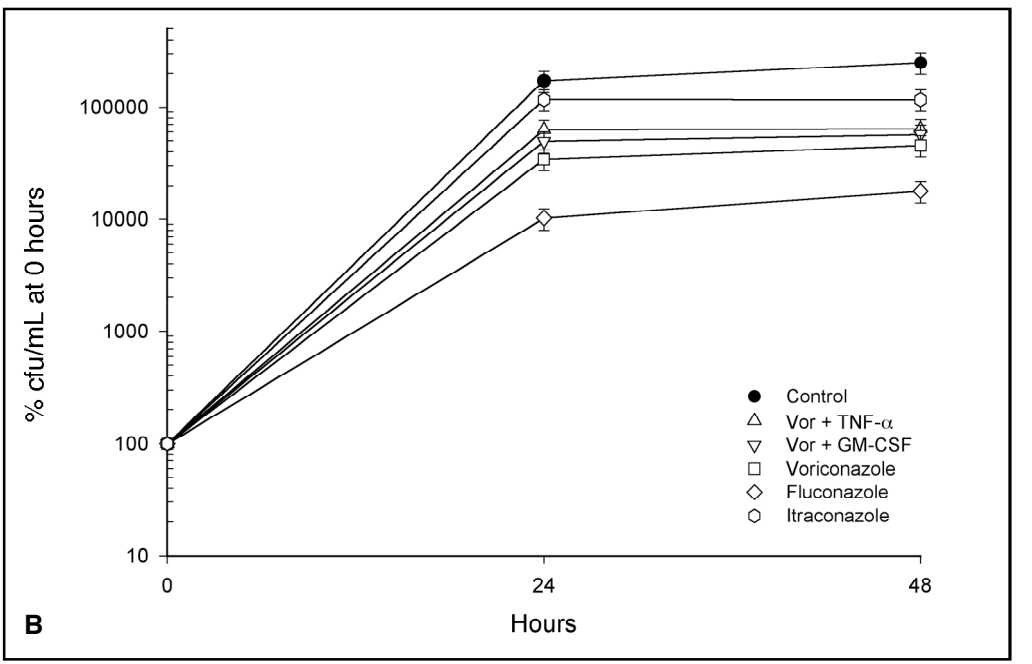
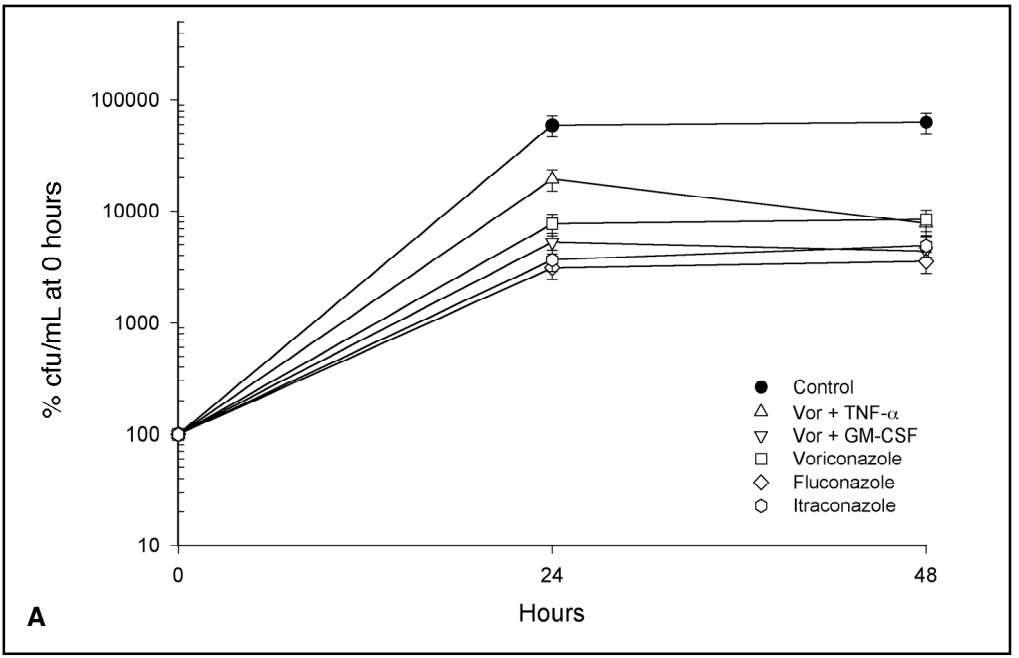




**Figure 1.** Anticandidal activity of voriconazole, itraconazole, and fluconazole at 1 x MIC against *Candida albicans* in human monocyte-derived macrophages. (A) $flu^R$ strain 8336-2; (B) $flu^R$ strain ATCC 64550.

following incubation of the plates at 37˚C for 24 h in an atmosphere containing 5% $CO_2$.

**Statistical Methods**

Each experiment described above was repeated at least three times. The analy-



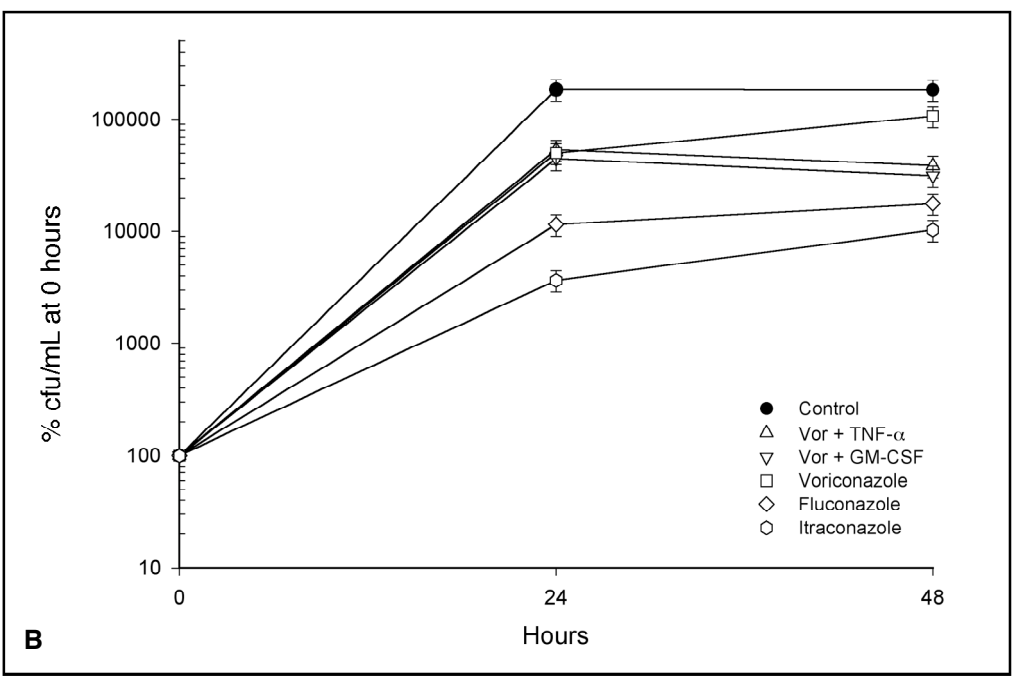




**Figure 2.** Anticandidal activity of voriconazole, itraconazole, and fluconazole at 1 x MIC against *Candida albicans* in human monocyte-derived macrophages. (A) flu$^S$ strain ATCC 90028; (B) flu$^S$ strain ATCC 56882.

sis of variance methodology[31] with the $\log_{10}$ transformation was used to analyze the data. For final presentation, average $\log_{10}$ values were converted to geometric mean values in the original units. Viable counts are expressed as percentages of the cfu/mL at zero hour. The standard errors of the geometric means (SEM)

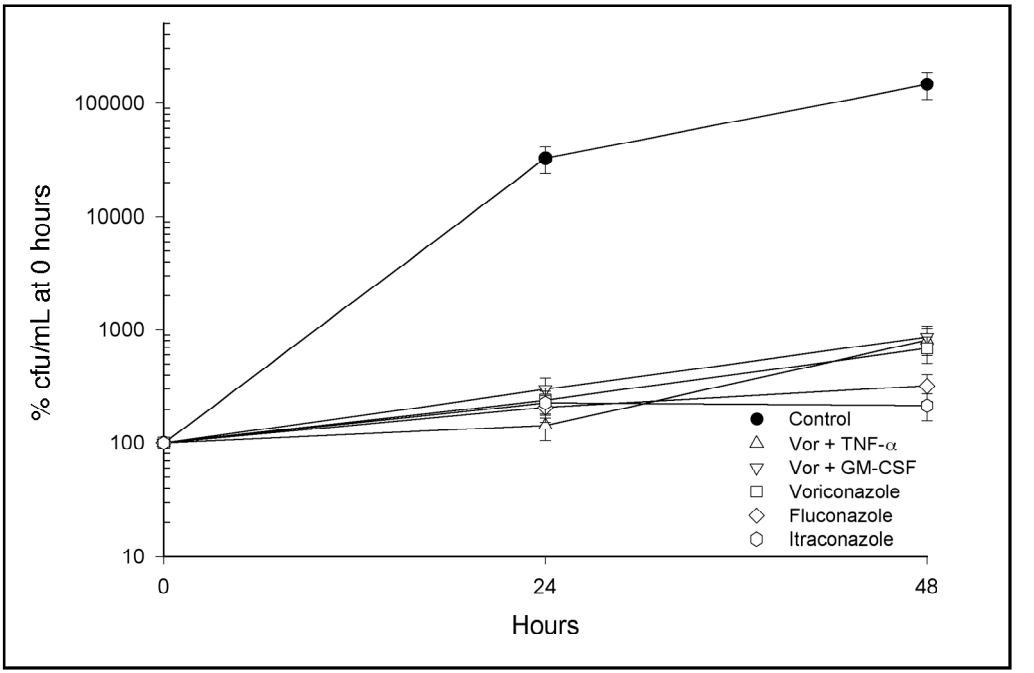



**Figure 3.** Anticandidal activity of voriconazole, itraconazole, and fluconazole at 1 x MIC against *Candida albicans* in human monocyte-derived macrophages: flu$^S$ strain T=6.

are depicted as error bars in Figures 1 through 3. Multiple contrasts were tested under the Bonferroni criteria.[32] Null hypotheses were specified a priori. The level of significance is 0.01.

## RESULTS

Figures 1 through 3 show the anticandidal activity of MDM with voriconazole in the presence and absence of GM-CSF or TNF-□, and the anticandidal activity of MDM with fluconazole and itraconazole in the absence of cytokines. Statistical analysis indicated that neither GM-CSF nor TNF-□] affected the intracellular anticandidal activity of MDM with itraconazole or fluconazole, and that the anticandidal activities of MDM activated only with GM-CSF or TNF-□ were similar to those of inactivated controls. Therefore, these data are not shown in the figure.

Figure 1A shows the anticandidal effects of MDM with voriconazole, itraconazole, and fluconazole at 1 x MIC, with and without cytokines (voriconazole only), against flu$^R$ *C albicans* 8336-2 in MDM. All azoles showed significant anticandidal activity compared with the control at 24 and 48 h ($P$<0.01). At 48 h voriconazole was more effective than itraconazole or fluconazole ($P$<0.01). However, at 24 h the effects of voriconazole and itraconazole were similar and greater than that of fluconazole ($P$<0.01). GM-CSF reduced the anticandidal effect of voriconazole at 24 h, while GM-CSF and TNF-□ reduced this effect at 48 h ($P$<0.01).

Figure 1B depicts the anticandidal effects of MDM with the three azoles at 1 x MIC, with and without cytokines (voriconazole only), against flu$^R$ *C albicans* ATCC 64550 in MDM. At both 24 and 48 h, fluconazole was most effective ($P$<0.01), and voriconazole was more effective than itraconazole ($P$<0.01). Although both fluconazole and voriconazole were more effective than the control ($P$<0.01), itraconazole was similar to the control. GM-CSF and TNF-□ did not affect the MDM antican-

**Table 1.** Production of GM-CSF and TNF-α by Human MDM Exposed to Azoles at Serum-Attainable Concentrations

| Azole | Geometric mean cytokine concentration in pg/mL (SEM) | |
|---|---|---|
| | GM-CSF | TNF-α |
| Voriconazole (2.5 mg/mL) | 2 (<1) | 10 (2) |
| Fluconazole (8.0 mg/mL) | 3 (<1) | 7 (2) |
| Itraconazole (0.4 mg/mL) | 4 (<1) | 4 (2) |
| Controls: | | |
| LPS (1 mg/mL) | 11 (3) | 1002 (281) |
| Untreated MDM | 10 (3) | 19 (5) |

didal activity with voriconazole.

Figure 2A shows the anticandidal activities of MDM with voriconazole, itraconazole, and fluconazole at 1 x MIC, with and without cytokines (voriconazole only), against flu$^S$ *C albi - cans* ATCC 90028 in MDM. At both 24 and 48 h, all azoles had similar anticandidal activity, which was significantly greater than that of the control ($P$<0.01). While GM-CSF had no effect on the anticandidal activity of voriconazole, anticandidal activity was decreased by TNF-α at 24 h but not at 48 h ($P$<0.01).

Figure 2B depicts the anticandidal activities of MDM with all three azoles at 1 x MIC, with and without cytokines (voriconazole only), against flu$^S$ *C albi - cans* ATCC 56882 in MDM. Although itraconazole was more effective than fluconazole at 24 h, their activities were similar at 48 h ($P$<0.01). Both itraconazole and fluconazole were more effective than the control ($P$<0.01), and voriconazole was similar to the control at 48 h but not at 24 h ($P$<0.01). At 24 h, voriconazole was the least effective antibiotic ($P$<0.01). However, both GM-CSF and TNF-α significantly increased the activity of voriconazole at 48 h ($P$<0.01). The increase was similar for both cytokines.

Figure 3 shows the MDM anticandidal activity of MDM with voriconazole, itraconazole, and fluconazole at 1 x MIC, with and without cytokines (voriconazole only), against flu$^S$ *C albi - cans* T=6 in MDM. At 24 h, the activities of voriconazole, itraconazole, and fluconazole were similar and were significantly greater than the control ($P$<0.01). However, at 48 h the activity of itraconazole was similar to that of fluconazole and was significantly greater than that of voriconazole ($P$<0.01). GM-CSF and TNF-α did not affect the anticandidal activity of MDM with voriconazole.

The production of GM-CSF and TNF-α by MDM following exposure to voriconazole, fluconazole, and itraconazole at serum-attainable levels is shown in Table 1. While LPS stimulated MDM production of TNF-α and GM-CSF, the effects of the three azoles at serum-attainable concentrations on the production of these two pro-inflammatory cytokines were negligible. Cytokine levels were similar to those of the unstimulated MDM controls.

## DISCUSSION

This study was undertaken in order to compare the intracellular anticandidal effects of three azoles in human MDM and to define the effects of GM-CSF and TNF-α on this anticandidal activity. The results clearly indicate that the

intracellular effects of voriconazole, itraconazole, and fluconazole studied at 1 x MIC in human MDM are variable and strain-dependent. For two of the five *C albicans* strains (ATCC 90028 and T=6), all azoles had similar activity at 24 h, although there were slight differences at 48 h. For strains ATCC 64550 and ATCC 56882, the most effective azoles were fluconazole and itraconazole, respectively, while voriconazole was most effective against strain 8336-2 ($flu^R$). Voriconazole was least effective against strain ATCC 56882 and itraconazole was least effective against strain ATCC 64550.

Previous studies have shown that fluconazole and voriconazole enter polymorphonuclear leukocytes.[10,11] The authors of this study are unaware of any study indicating that azoles also penetrate into human monocytes. Our data indicate that all of the azoles studied have intracellular anticandidal activity in human MDM. However, this activity varies with the azole, *C albicans* strain, and duration of infection in MDM. An earlier study that used different methodology and single strains of $flu^S$ and $flu^R$ *C albicans* showed that the anticandidal effect of voriconazole was greater than that of fluconazole in both monocytes and neutrophils, and that activation of these phagocytes with GM-CSF increased the intracellular effect of voriconazole.[18] In two other studies that used GM-CSF-activated neutrophils, fluconazole, and *C albicans,* the intracellular activity of the phagocytes was shown to be strain-dependent.[15,16] This study using human monocytes, three different azoles, and five strains of *C albicans* showed that although all three azoles increased the intracellular anticandidal activity of the MDM, this increase was azole- and strain-dependent. Furthermore, activation of MDM with GM-CSF or TNF-α did not affect the anticandidal activity of MDM in the presence of itraconazole or fluconazole when 1 x MIC of these azoles was used. In contrast, in the presence of voriconazole and cytokines, a decrease in MDM activity was observed in two *C albicans* strains (GM-CSF and TNF-α for 8336-2 and TNF-α for ATCC 90028), while an increase in the MDM activity was observed on ATCC 56882 with GM-CSF and TNF-α, and no effect was observed on strains ATCC 64550 and T=6.

The effects of azoles on cytokine production by MDM were determined in order to ensure that any effects of added cytokines were attributable to the added cytokines and did not result from cytokine production stimulated by the azoles. These results indicate that the effects of voriconazole, fluconazole, and itraconazole on production of GM-CSF and TNF-α by human MDM were negligible. Cytokine levels were similar to those of unstimulated MDM controls.

In conclusion, by studying five different strains of *C albicans*, both $flu^S$ and $flu^R$, and three azoles, these data clearly show that the intracellular anticandidal effects of human MDM cannot be predicted and that they are *C albicans* strain- and azole-dependent. *C albicans* strains may need to be evaluated individually in order to select the azole with the greatest intracellular anticandidal activity. The anticandidal effect of cytokine-activated MDM in the presence of voriconazole was strain-dependent and could be increased, decreased, or remain unchanged. Cytokines did not affect the anticandidal activity of MDM in the presence of fluconazole or itraconazole.

## ACKNOWLEDGMENT

This work was supported by Pfizer Pharmaceuticals and, in part, by the Office of Research and Development of the Department of Veterans Affairs. We thank Stephanie Brennan and Chendell Sheehan for their most valuable secretarial assistance.

## REFERENCES


1. Beck-Sague CM, Jarvis W. National nosocomial infections surveillance system. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. *J Infect Dis.* 1993;167:1247-1251.
2. Lewis RE, Klepser ME. The changing face of nosocomial candidemia: epidemiology, resistance, and drug therapy. *Am J Health Syst Pharm.* 1999;56:525-535.
3. Pfaller MA. Nosocomial candidiasis: emerging species, reservoirs, and modes of transmission. *Clin Infect Dis.* 1996;22(suppl 2):S89-S94.
4. Barry AL, Brown SD. In vitro studies of two triazole antifungal agents (voriconazole [UK-109,496] and fluconazole) against *Candida* species. *Antimicrob Agents Chemother.* 1996;40:1948-1949.
5. Belanger P, Nast CC, Fratti R et al. Voriconazole (UK-109,496) inhibits the growth and alters the morphology of fluconazole-susceptible and -resistant *Candida* species. *Antimicrob Agents Chemother.* 1997;41:1840-1842.
6. Cuenca-Estrella M, Díaz-Guerra TM, Mellado E, et al. Comparative in vitro activity of voriconazole and itraconazole against fluconazole-susceptible and fluconazole-resistant clinical isolates of *Candida* species from Spain. *Eur J Clin Microbiol Infect Dis.* 1999;18:432-435.
7. Girmenia C, Tuccinardi C, Santilli S, et al. In vitro activity of fluconazole and voriconazole against isolates of *Candida albicans* from patients with hematological malignancies. *J Antimicrob Chemother.* 2000;46:479-483.
8. Lee JK, Peters D, Obias AA, et al. Activity of voriconazole against *Candida albicans* and *Candida krusei* isolated since 1984. *Int J Antimicrob Agents.* 2000;16:205-209.
9. Marr KA. Empirical antifungal therapy—new options, new tradeoffs. *N Engl J Med.* 2000;346:278-280.
10. Pascual A, Garcia I, Conejo C, et al. Uptake and intracellular activity of fluconazole in human polymorphonuclear leukocytes. *Antimicrob Agents Chemother.* 1993;37:187-190.
11. Balesta S, Garcia I, Perea EJ, et al. Uptake and intracellular activity of voriconazole in human polymorphonuclear leukocytes. *J Antimicrob Chemother*. 2005;55:785-787.
12. Calderone R, Sturtevant J. Macrophage interactions with *Candida. Immunol Ser.* 1994;60:505-515.
13. Baltch AL, Smith RP, Franke MA, et al. Effects of cytokines and fluconazole on the activity of human monocytes against *Candida albicans*. *Antimicrob Agents Chemother.* 2001;45:96-104.
14. Lenhoff S, Rosberg B, Olofsson T. Granulocyte interactions with GM-CSF and G-CSF secretion by endothelial cells and monocytes. *Eur Cytokine Netw.* 1999;10:525-532.
15. Natarajan U, Randhawa N, Brummer E, et al. Effect of granulocyte-macrophage colony-stimulating factor on candidacidal activity of neutrophils, monocytes or monocyte-derived macrophage and synergy with fluconazole. *J Med Microbiol.* 1998;47:359-363.
16. Natarajan U, Brummer E, Stevens DA. Effect of granulocyte colony-stimulating factor on the candidacidal activity of polymorphonuclear neutrophils and their collaboration with fluconazole. *Antimicrob Agents Chemother.* 1997;41:1575-1578.
17. Smith PD, Lamerson CL, Banks SM, et al. Granulocyte-macrophage colony-stimulating factor augments human monocyte fungicidal activity for *Candida albicans. J Infect Dis.* 1999;161:999-1005.
18. Vora S, Purimetla N, Brummer E, et al. Activity of voriconazole, a new triazole, combined with neutrophils or monocytes against *Candida albicans*: effect of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. *Antimicrob Agents Chemother.* 1998;42:907-910.
19. Yamamoto Y, Klein TW, Tomioka M, et al. Differential effects of granulocyte/macrophage colony-stimulating factor (GM-CSF) in enhancing macrophage resistance to *Legionella pneumophila* vs. *Candida albicans*. *Cell Immunol*. 1997;76:75-81.
20. Nemunaitis J, Buckner CD, Dorsey KS, et al. Retrospective analysis of infectious disease in patients who received recombinant human granulocyte-macrophage colony-stimulating factor versus patients not receiving a cytokine who underwent autologous bone marrow transplantation for treatment of lymphoid cancer. *Am J Clin Oncol.* 1998;21:341-346.
21. Poynton CH, Barnes RA, Rees J. Interferon-□ and granulocyte-macrophage colony-stimulating factor for the treatment of hepatosplenic candidosis in patients with acute leukemia. *Clin Infect Dis.* 1998;26:239-240.
22. Louie A, Baltch AL, Smith RP, et al. Tumor necrosis factor alpha has a protective role in a murine model of systemic candidiasis. *Infect Immun.* 1994;62:2761-2772.
23. Sabo JA, Abdel-Rahman SM. Voriconazole: a new triazole antifungal. *Ann Pharmacother.* 2000;34:1032-1043.

24. Li R-K, Ciblak MA, Nordoff N, et al. In vitro activities of voriconazole, itraconazole, and amphotericin B against *Blastomyces dermatitidis, Coccidioides immitis* and *Histoplasma capsulatum. Antimicrob Agents Chemother.* 2000;44:1734-1736.

25. Sanati H, Belanger P, Fratti R, et al. A new triazole, voriconazole (UK-109,496), blocks sterol biosynthesis in *Candida albicans* and *Candida krusei*. *Antimicrob Agents Chemother.* 1997;41:2492-2496.

26. Sheehan DJ, Hitchcock CA, Sibley CM. Current and emerging azole antifungal agents. *Clin Microb Rev.* 1999;12:40-79.

27. Walsh TJ, Pappas P, Winston DJ, et al. Voriconazole compared with liposomal amphotericin B for empirical antifungal therapy in patients with neutropenia and persistent fever. *N Engl J Med.* 2002;346:225-234.

28. Andriole VT. Current and future antifungal therapy: new targets for antifungal agents. *J Antimicrob Chemother.* 1999;44:151-162.

29. National Committee for Clinical Laboratory Standards. *Reference Method for Broth Dilution Antifungal Susceptibility Testing for Yeasts: Approved Standard.* Wayne, PA: National Committee for Clinical Laboratory Standards; 2002. NCCLS Document M27-A2.

30. Rex JH, Pfaller MA, Walsh TJ, et al. Antifungal susceptibility testing: practical aspects and current challenges. *Clin Microb Rev.* 2001;14:643-658.

31. Stuart A, Ord JK. *Kendall's Advanced Theory of Statistics.* New York, NY: Oxford University Press; 1991;2:1101-1153.

32. Fleiss JL. *The Design and Analysis of Clinical Experiments*. New York, NY: John Wiley & Sons; 1986:103.