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Uptake and Intracellular Activity of Fluconazole in Human Polymorphonuclear Leukocytes

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The penetration of fluconazole into human polymorphonuclear leukocytes (PMNs) and tissue culture epithelial cells (McCoy) was evaluated. At different extracellular concentrations (0.5 to 10 mg/liter), fluconazole reached cell-associated concentrations greater than the extracellular ones in either human PMNs (intracellular concentration to extracellular concentration ratio, ≥2.2) or McCoy cells (intracellular concentration to extracellular concentration ratio, ≥1.3). The uptake of fluconazole by PMNs was rapid and reversible but was not energy dependent. The intracellular penetration of fluconazole was not affected by environmental pH or temperature. Ingestion of opsonized zymosan and opsonized *Candida albicans* did not significantly increase the amount of PMN-associated fluconazole. At therapeutic extracellular concentrations, the intracellular activity of fluconazole against *C. albicans* in PMNs was significantly lower than that of amphotericin B. It was concluded that fluconazole reaches high intracellular concentrations within PMNs but shows moderate activity against intracellular *C. albicans* in vitro.

Candida spp. are one of the most prominent fungal pathogens that cause severe infections in immunosuppressed patients. Polymorphonuclear leukocytes (PMNs) are an important component of the host defense mechanisms against *Candida* spp. infections. The effect of antifungal agents on PMN activity could be an important factor in the treatment of these infections, particularly in immunosuppressed patients.

Fluconazole is a novel antifungal agent with a bistriazole structure that is remarkably different from other azole antifungal agents; for example, it has excellent penetration into cerebrospinal fluid and various tissues (1, 8). The pharmacokinetic characteristics of this drug explain the successful use of fluconazole in the treatment of both superficial and deep-seated infections (10, 13).

At therapeutic concentrations, fluconazole does not affect different functions of human phagocytes in vitro, as has been observed by different investigators (4, 9). There is not much information, however, on the ability of this antifungal agent to penetrate and concentrate within phagocytic and nonphagocytic cells.

The purpose of the present study was to evaluate the intracellular penetration of fluconazole into human PMNs and tissue culture cells. The possible mechanism involved in the membrane transport of this antimicrobial agent was also evaluated. Finally, the correlation between the intracellular penetration and activity against *Candida albicans* within human PMNs was also assessed.

MATERIALS AND METHODS

Isolation of PMNs. PMNs were recovered from heparinized venous blood of healthy donors and were purified by previously described methods (7). PMN preparations were 97% pure. Final cell suspensions were adjusted to 5×10^6 PMNs/ml in Hanks' balanced salt solution. The PMNs were 95% viable by trypan blue exclusion.

Tissue culture cells. Tissue culture epithelial cells obtained from human synovial fluid (McCoy cells; Flow Laboratories, Irvine, Calif.) were grown in minimal essential medium (Flow) supplemented with 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Flow) and containing 10% fetal calf serum (Flow) without antibiotics. For each experiment, cells were detached from tissue culture bottles with trypsin-EDTA (Flow) and were suspended in Hanks' balanced salt solution at a concentration of 5×10^6 cells per ml.

Fluconazole uptake by cells. Uptake of radiolabeled fluconazole was determined by means of a velocity gradient centrifugation technique described by Klempner and Styrt (3). [^{3}H]fluconazole (specific activity, about 20 Ci/mmol; Pfizer Central Research, Sandwich, United Kingdom) was used in the study. In these experiments, PMNs or McCoy cells were incubated in Hanks' balanced salt solution containing different concentrations of fluconazole. After different incubation periods, the cells were separated from the extracellular solution by centrifugation through a water-impermeable silicone-oil barrier in a microcentrifuge tube. A 10-μl aliquot of the extracellular medium and the entire cell pellet, obtained by cutting off the portion of the microcentrifuge tube containing the pellet, were placed in 3 ml of scintillation fluid (Ready Microb; Beckman Instruments) and counted in a liquid scintillation counter (model LS 1701; Beckman Instruments).

The intracellular water space was measured by using tritiated water and the extracellular marker [^{14}C]polyethylene glycol (^3H_2O, 1.0 mCi/g; [^{14}C]polyethylene glycol, 1.4 mCi/g [Du Pont, NEN Research Products, Boston, Mass.]). The PMNs were incubated with these radiolabeled compounds for 20 min at 37°C, and then the cells were separated from the extracellular fluid by velocity gradient centrifugation as described above and counted in a liquid scintillation counter (3). The total water content of the PMN pellet was corrected for trapped extracellular water, i.e., polyethylene glycol space, to obtain the intracellular water space. From the values obtained from this procedure, PMN-associated antimicrobial agent concentrations were calculated and expressed as a ratio of the intracellular concentration to the extracellular concentration (C/E) (3).

Characterization of fluconazole uptake. Further studies

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were performed as described previously (5) to elucidate the mechanism of fluconazole uptake by PMNs. The effect of cell viability was studied by using PMNs killed by exposure to 10% formalin for 30 min. These cells were then washed and resuspended in fresh medium. Moreover, the influences of environmental temperature, pH, metabolic inhibitors, and potential competitive inhibitors were evaluated. The influence of temperature was examined by comparing antibiotic uptake at 4 and 37°C. The pH profile of fluconazole uptake in medium preadjusted to different external pH values (5, 7.2, and 9) by the addition of 10 N HCl or 10 N NaOH was measured. An inhibitor of glycolysis (sodium fluoride, 1.5×10^{-3}M; Sigma Chemical Co., St. Louis, Mo.), an inhibitor of mitochondrial oxidative metabolism (sodium cyanide, 1.5×10^{-3}M; Sigma), a blocker of the proton gradient (carbonyl cyanide m-chlorophenylhydrazone, 1.5×10^{-5}M; Sigma), and an uncoupler of oxidative phosphorylation (2,4-dinitrophenol, 1×10^{-4}M; Sigma) were used as metabolic inhibitors. The PMNs in Hanks balanced salt solution with or without metabolic inhibitors were incubated for 30 min at 37°C (5). Fluconazole (final concentration, 2 mg/liter was then added, and the uptake was measured as described above.

The efflux or reversibility of binding of PMN-associated fluconazole was also studied. The PMNs were incubated for 10 min at 37°C with fluconazole, collected by centrifugation, and rapidly suspended in fluconazole-free medium (5). PMN-associated fluconazole was quantitated at various time intervals (5, 10, and 20 min) after removal of the extracellular fluconazole.

Organism and susceptibility to antifungal agents. *C. albicans* YO1-09 was kindly supplied by Pfizer Central Research (Sandwich, United Kingdom). Susceptibility studies were determined by a dilution technique with HR medium (Pfizer). The MICs of fluconazole (Pfizer) and amphotericin B (Squibb) were 1.5 and 0.25 mg/liter, respectively.

Intracellular activity of fluconazole. To evaluate the intracellular activity of antifungal agents, a modification of a previously described method was used (6). Briefly, after preopsonization of *C. albicans* blastospores in 10% pooled human serum (15 min, 37°C), 0.1 ml of yeast suspension (2×10^7 CFU/ml) and 0.1 ml of phagocytes (2×10^6 PMNs/ml) were combined in a series of polypropylene biovials (Beckman Instruments) and incubated in a shaker (250 rpm) for 60 min at 37°C. Subsequent to incubation, the mixtures were washed three times with 3 ml of ice-cold phosphate-buffered saline by using differential centrifugation to remove extracellular blastospores. Cells were then resuspended in 0.2 ml of RPMI medium. At that time (designated time zero), essentially all yeasts were cell associated, as evaluated by light microscopy. The different antifungal agents were added at different concentrations (0.5, 2, and 10 mg/liter), and the vials were reincubated in a shaker (250 rpm) at 37°C. Vials were removed at time zero (control) and after 3 h of incubation (control and samples with antifungal agents), and the phagocytes were lysed in distilled water (15 min, room temperature). Finally, the samples were diluted and placed onto Sabouraud agar plates and the CFU was counted after the plates were incubated at 37°C for 2 days. All samples were processed in duplicate. The data are expressed as the percentage of surviving *C. albicans* in comparison with the levels in controls (without antifungal agent). In addition to determining fungal survival, morphologic studies were also routinely performed at time zero and after 3 h of incubation to evaluate the disposition of yeasts (cell associated or extracellular). Samples of 50 μl were removed from biovials and deposited on glass slides by using a cytocentrifuge (Cytospin 2; Shandon Southern Instruments). After staining with Wright's stain, samples were examined by light microscopy.

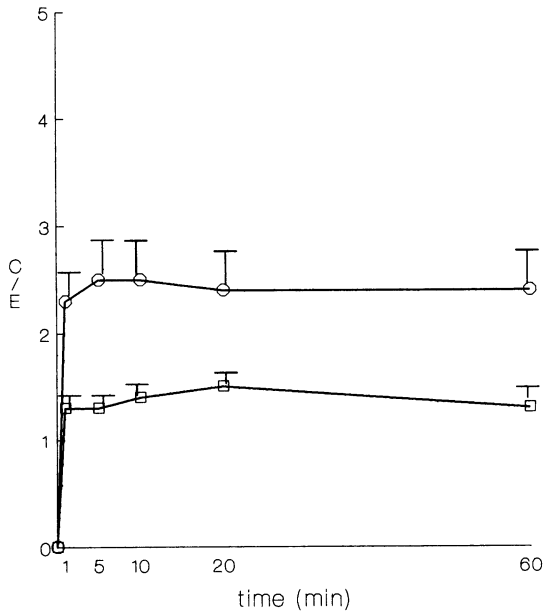

FIG. 1. Fluconazole uptake by human PMNs (○) and McCoy cells (□) at different periods of time ($n = 6$). Extracellular fluconazole concentration, 2 mg/liter.

Statistical analysis of data. Data are expressed as means ± standard deviations. Differences among groups were compared by analysis of variance, which was used to assess statistical significance at $P < 0.05$.

RESULTS

Uptake of fluconazole by human PMNs and McCoy cells. Figure 1 shows the C/E ratios of fluconazole in human PMNs and McCoy cells at different times. Fluconazole penetrates into PMNs rapidly, reaching intracellular concentrations twice as great as the extracellular ones. The C/E ratios of fluconazole in McCoy cells were lower than those observed in PMNs, but they were still greater than 1.

The effects of different extracellular concentrations in the uptake of fluconazole by PMNs are shown in Fig. 2. The C/E ratio values were greater than 2 at extracellular concentrations ranging from 0.5 to 10 mg/liter.

To evaluate whether the fluconazole that had been taken up by PMNs was tightly bound to cellular components, we studied the kinetics of efflux (Fig. 3). The reversibility of binding of fluconazole was rapid, with 83% of the cell-associated drug being lost by 5 min (compare values at 10 and 15 min in Fig. 3).

The effects of cell viability, environmental temperature, pH, and different metabolic inhibitors on fluconazole uptake by PMNs are shown in Table 1. The intracellular penetration of fluconazole was significantly higher when formalin-killed cells were used. None of the other conditions affected the C/E ratio values of this drug.

Finally, we evaluated the influence of phagocytosis and other cell membrane stimuli on the uptake of fluconazole. We found that ingestion of opsonized *C. albicans* (C/E ratio,

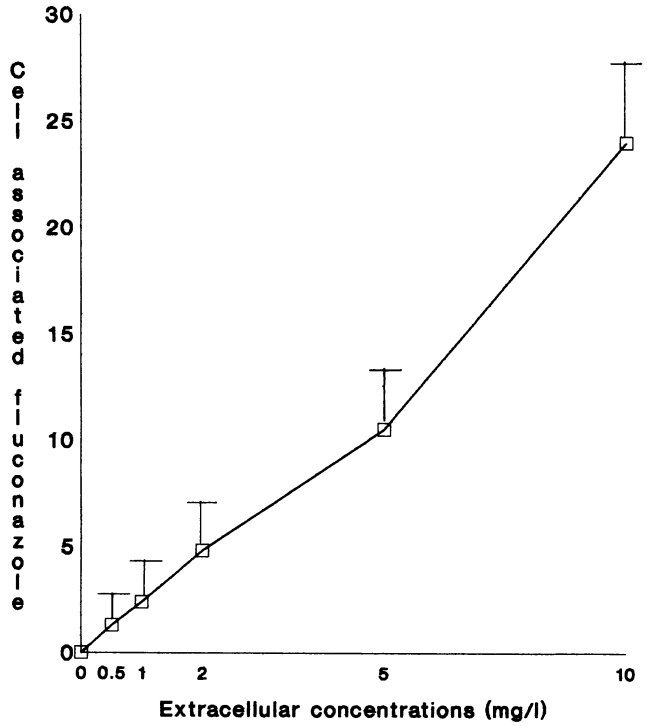

FIG. 2. PMN-associated fluconazole (in milligrams per liter) at different extracellular concentrations (n = 5). Experiments were carried out for 20 min.

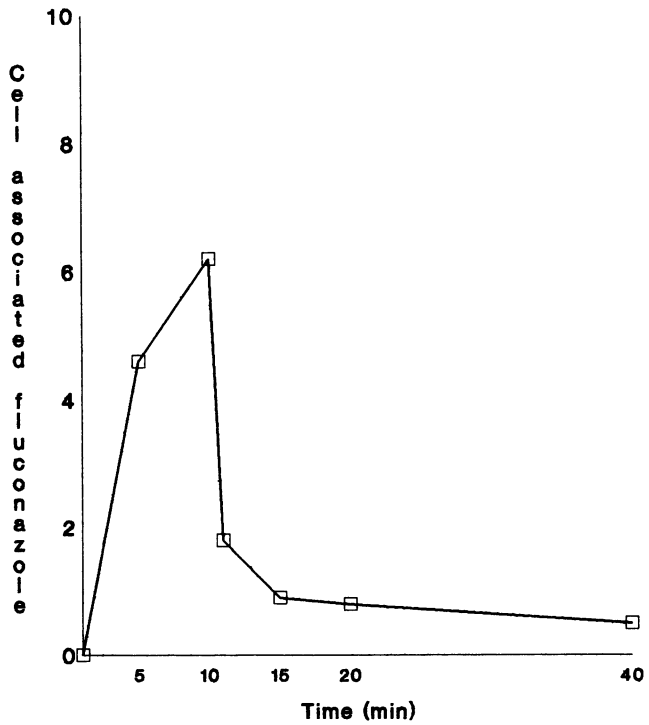

FIG. 3. Reversibility of binding of PMN-associated fluconazole (in milligrams per liter) after removal of the extracellular drug. Data are means of three experiments at each time point. Cells were centrifuged and resuspended in drug-free medium after fluconazole accumulation for 10 min.

2.7 ± 0.7) or zymosan (C/E ratio, 2.2 ± 0.4) and stimulation with phorbol myristate acetate (C/E ratio, 2.4 ± 0.3) did not affect the penetration of fluconazole into human PMNs (control C/E ratio, 2.5 ± 0.5).

Intracellular activity of fluconazole. The intracellular activity of fluconazole compared with that of amphotericin B against *C. albicans* was evaluated in a 3-h assay (Fig. 4). None of the evaluated concentrations of fluconazole significantly affected the intracellular survival of *C. albicans* blastospores. Amphotericin B, however, showed significant intracellular activity at concentrations of 2 and 10 mg/liter.

As determined by light microscopy, amphotericin B completely blocked germ tube formation of *C. albicans* in human PMNs. Fluconazole, however, incompletely inhibited germ tube formation inside of these phagocytes.

DISCUSSION

The intracellular penetration and activity of fluconazole within phagocytic cells could be an important factor in the treatment of severe fungal infections, especially in immunosuppressed patients.

The results presented above indicate that fluconazole penetrates into human PMNs rapidly. Fluconazole reaches concentrations in PMNs that are at least twice as great as that in the surrounding medium. The penetration of this imidazole antifungal agent was not affected by pH, environmental temperature, or different metabolic inhibitors. The accumulation of fluconazole does not require cell viability and is actually enhanced by exposure of cells to formaldehyde. The influence of phagocytosis and cell membrane stimuli on the uptake of fluconazole was also evaluated. Neither the phagocytosis of opsonized *C. albicans* and opsonized zymosan nor the stimulation of PMNs with phorbol myristate acetate affected the PMN-associated fluconazole concentration. These data indicate that the uptake of fluconazole by PMNs occurs by a passive mechanism probably owing to its high lipid solubility.

The fluconazole that becomes cell associated under the conditions of the present study is still recoverable in a fully active form. A similar phenomenon has been described for other antimicrobial agents such as fluoroquinolones (11).

The penetration of fluconazole into cultured epithelial cells was lower than that observed in PMNs, but it still reached concentrations slightly greater than the extracellular ones. The use of the model described here offers an inter-

TABLE 1. Effects of cell viability, environmental temperature, pH, and metabolic inhibitors on intracellular penetration of fluconazole in human PMNs (n = 6)[a]

Condition	C/E ratio
Viable cells at 37°C	2.6 ± 0.4
Viable cells at 4°C	2.8 ± 0.6
Dead cells at 37°C	4.3 ± 1.1[b]
pH 6	2.2 ± 0.2
pH 7	2.4 ± 0.5
pH 8	2.1 ± 0.4
Sodium fluoride	3.1 ± 0.6
Sodium cyanide	2.8 ± 0.5
Carbonyl cyanide *m*-chlorophenylhidrazone	2.1 ± 0.4
2,4-Dinitrophenol	2.2 ± 0.7

[a] Experiments were carried out for 20 min with extracellular fluconazole concentrations of 2 mg/liter.

[b] $P < 0.05$.

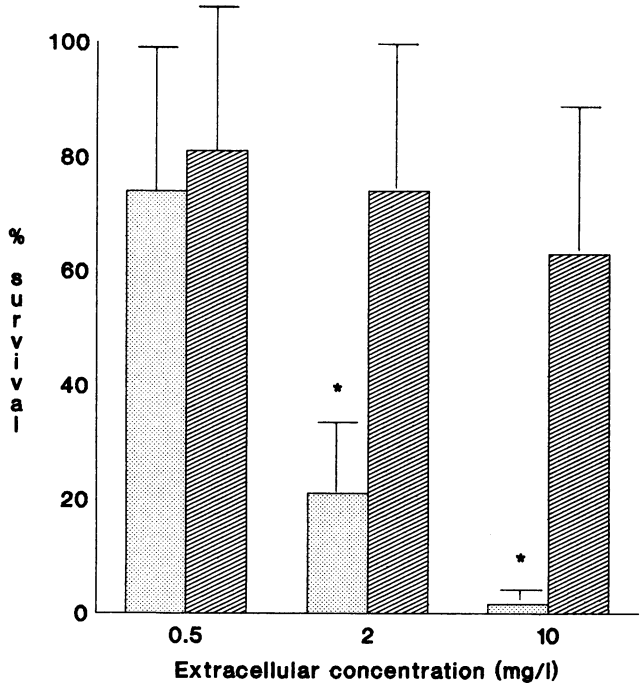

FIG. 4. Effects of amphotericin B (▩) and fluconazole (▨) on killing of *C. albicans* ingested by human PMNs. Data are expressed as percentages of the levels in controls (without antifungal agent) ($n = 5$). *, $P < 0.05$.

esting insight into the penetration of antifungal agents into nonphagocytic cells and could have relevance to infections produced by the fungi that can invade these types of cells.

The contrast between intracellular penetration and activity has been described for a few antimicrobial agents (6). At extracellular concentrations of between 0.5 and 10 mg/liter, fluconazole did not affect the intracellular destruction of *C. albicans* blastospores by human PMNs. At these same concentrations, fluconazole inhibited germ tube formation inside PMNs, although it did so incompletely. Amphotericin B at 2 and 10 mg/liter, however, significantly enhanced the intracellular destruction of *C. albicans*. Moreover, amphotericin B completely blocked germ tube formation of *C. albicans* in PMNs. Similar results have been described with both antifungal agents in macrophages (12).

Since it has been demonstrated that *C. albicans* may resist intracellular killing by phagocytes through the formation of germ tubes, antifungal agents that inhibit intracellular germ tube formation could therefore facilitate the host defense against *C. albicans*. For this reason, it has been suggested that an in vitro test that incorporates the mycelial form might provide a better indication of the therapeutic value of the imidazole antifungal agents than a test in which the inoculum is the blastospore form (2, 12). The efficacy of fluconazole in vivo seems to exhibit a better correlation with its in vitro activity against germ tube formation than it does with the effect of this drug on the number of blastospores (2). Moreover, the inhibition of germ tube formation by fluconazole could facilitate the anticandidal activity of human phagocytes.

In summary, fluconazole penetrates rapidly into human PMNs by a possible passive mechanism that allows fluconazole to reach concentrations at least double the extracellular ones. The intracellular activity of fluconazole against intracellular *C. albicans* blastospores was significantly lower than that of amphotericin B.

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