Ketoconazole, Amphotericin B, and Amphotericin B Methyl Ester: Comparative In Vitro and In Vivo Toxicological Effects on Neutrophil Function

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We investigated a number of parameters for host defense after the in vitro addition of the antifungal agents ketoconazole, amphotericin B (AMB), and amphotericin B methyl ester (AME). Similar assays were repeated before and after patients received the former two drugs. Viability by trypan blue exclusion, adherence by nylon wool columns, chemotaxis by the under-agarose technique, phagocytosis and killing by chemiluminescence, colony counts, and acridine orange direct visualization were assayed. In striking contrast to AMB and AME, ketoconazole demonstrated no significant effect on neutrophils. Adherence in the presence of therapeutic plasma levels of AMB and AME was decreased ($P \leq 0.005$) at low drug concentrations, whereas at higher concentrations, adherence was increased ($P < 0.001$). The chemotactic responses of cells incubated with AMB and AME demonstrated marked suppression. Phagocytic capacity and killing were decreased ($P \leq 0.005$) with AMB as compared with control assays and assays performed in the presence of ketoconazole and AME. However, no differences were observed between two patients who received AMB and two others treated with ketoconazole.

Many investigators have shown that not only do natural deficiencies and abnormalities alter the immunological responses, but such alterations can also be attributed to the toxicity of various antibiotics used in chemotherapy (7, 8, 14, 15).

The major chemotherapeutic agent for systemic mycoses is amphotericin B (AMB), a polyene antibiotic. It is well documented that treatment with this antifungal drug is complicated by a variety of toxic effects to the patient (4, 17). Amphotericin B methyl ester (AME) is a water-soluble derivative of AMB first prepared by Mechlinski and Schaffner (16, 23). It is significantly less toxic than its parent drug in mice and dog models (9–11). However, more recent results in humans have raised some questions concerning possible toxic effects on the central nervous system (P. D. Hoeprich, M. M. Kawa-ichi, K. K. Lee, and C. P. Schaffner, Program Abstr. Intersei. Conf. Antimicrob. Agents Chemother. 19th, Boston, Mass., abstr. no. 157, 1979).

The imidazole derivative ketoconazole is an oral, broad-spectrum antifungal drug that has no major side effects and appears to be a potentially valuable agent for antifungal therapy.

There has been no comprehensive in vitro study investigating the comparative effects of these compounds on neutrophil function. To evaluate the competency of neutrophils, cells were exposed to ketoconazole, AMB, and AME at various concentrations and examined with assay systems to assess viability, adherence, chemotaxis, phagocytosis, and killing. In addition, four patients receiving antifungal agents were examined for changes in neutrophil capacity.

MATERIALS AND METHODS

Separation of peripheral blood leukocytes. Fresh heparinized whole blood (50 U/ml) was obtained on each experimental day from healthy volunteer donors or from patients before and during their antifungal therapy. Patients receiving antifungal chemotherapy were monitored so that the phlebotomy was performed during peak drug concentrations. Leukocytes were procured by adding 2 ml of Dextran 70 (Phar-macia Fine Chemicals, Inc., Piscataway, N.J.) to each
10 ml of blood, incubating at 37°C for 60 min, and then removing the leukocyte-rich supernatant fluid. Ficoll–
Hypaque centrifugation was used to isolate pure populations of neutrophils. The cells were suspended in a
hypotonic solution of 0.84% ammonium chloride to lyse erythrocyte contaminants. After the cells were
washed twice with Gey balanced salt solution, the neutrophils were adjusted to an appropriate concentra-
tion for the particular assay.

**Antifungal agents.** Ketoconazole (Janssen R & D, Inc., New Brunswick, N.J.) was dissolved in sterile
acidified water (pH 2.3) as the initial stock suspension. Subsequent dilutions to obtain final concentrations ranging from 1 to 50 μg/ml were made with either Gey balanced salt solution, Hanks balanced salt solution, or whole blood.

AMB was purchased as a sterile, lyophilized, deoxycholate-stabilized, phosphate-buffered powder (Fung-
zone; E. R. Squibb & Sons, Princeton, N.J.), and AME aspartate was kindly supplied by Patrick A. Diassi, Squibb Institute for Medical Research, Princeton, N.J. These products were reconstituted in sterile distilled water, and dilutions were prepared fresh as mentioned above. Ketoconazole, AMB, and AME were added to either heparinized whole blood or washed cell suspensions and incubated for a minimum of 60 min before the various assays were performed. Control, or untreated, neutrophil suspensions were preincubated in buffer for the same duration of time.

The cultures, obtained 1 h after completion of intravenous infusion (mean value, 1.21 μg/ml) (6). Blood from all patients was drawn during peak therapeutic drug levels, and no additional antifungal agents were added to the whole blood or neutrophil suspensions collected.

**Cell viability.** Whole blood and washed neutrophil suspensions were exposed for 3 h to various concentra-
tions of each antifungal agent. Viability was assessed by the ability of the cells to exclude the vital
dye trypan blue (21). At 30-min intervals, samples of the whole blood or washed neutrophils were mixed
with equal volumes of 0.4% trypan blue stain (GIBCO Laboratories, Grand Island, N.Y.). A total of 200 cells
were counted microscopically, and the percent viability was determined.

**Neutrophil adherence.** Adherence was measured with nylon fiber columns as previously described (13).

Briefly, 50 mg of nylon fiber was carefully packed into a Pasteur pipette to a measured length of 16 mm. A 1-
ml amount of blood was dispensed into the column and allowed to filter through the nylon fiber by gravity.
The precolumn neutrophil count and differential were compared with the count of the effluent blood to
calculate the percentage of neutrophils adhering to the column. The percentage of adherent neutrophils
was calculated as follows: adherence = 100% – [(neu-

trophils per milliliter in effluent sample/neutrophils per milliliter in original sample) × 100].

Chemotaxis. Neutrophils treated with ketocona-

zole, AMB, or AME in a serum-free system were assessed for their chemotactic responses to zymosan-

activated autologous serum as previously described (18). The agarose solution was dispensed into a sterile
tissue culture dish (60 by 15 mm) and allowed to harden. Three wells, with a diameter of 2 mm and spaced 2.5 mm apart, were cut. Each plate contained six series of three wells. A 10-μl amount of the cell suspension (10⁶ neutrophils per ml), preincubated with the antifungal agents, was added to the middle wells in three of the six sets in the agarose plates. The remaining three middle wells were filled with 10 μl of control cell suspensions incubated in buffer. The six outer wells were all filled with 10 μl of activated autologous serum, and the inner wells were filled with

10 μl of buffer. A 2-h incubation period was carried out at 37°C in a humidified atmosphere containing 5% CO₂
in air. Plates were then fixed, and quantitation was determined by counts of migrated cells in a single
plane (19) and expressed as a percentage of the control.

Chemiluminescence. Procedures for serum opson-

ization of zymosan particles and for the measure-

ment of the chemiluminescence have been described in detail previously (1). A beta-counting spectrometer

was adjusted for out-of-coincidence. Dark-adapted

polyethylene vials were filled with 4 ml of Gey bal-

anced salt solution containing 10⁶ neutrophils per ml and the appropriate concentration of the antifungal
agent. The vials were counted in sequence to obtain background measurements. A 1-ml amount of opso-

nized zymosan particles was added to each vial at zero
time, and each vial was counted for 15 s. All vials were counted sequentially until the peak chemilumin-
encescence response could be determined. The peak chemilu-

minescence of neutrophils treated with the antifun-
gal agents was expressed as a percentage of the peak chemiluminescence produced by control, untreated

neutrophils from the same donor.

**Preparation of bacteria (Staphylococcus au-

reus).** 502A stock cultures, stored at −70°C in Tryp-
ticase soy broth (BBL Microbiology Systems, Cock-
eysville, Md.) with glycerol, were thawed and inocu-
lated into 10 ml of Trypticase soy broth and incubated
for 18 h at 37°C. The cultures were centrifuged and

washed with Hanks balanced salt solution and ad-
justed to 1 × 10⁶ to 5 × 10⁸ bacteria per ml. They

contained 10% autologous serum.

**Phagocytic bacterial assay.** Phagocytosis and killing of bacteria were first measured using the bac-
tericidal method (22). Briefly, each 1-ml incubation mixture included 5 × 10⁹ colony-forming units of bac-
teria. The suspensions contained 10% autologous se-
rum with or without ketoconazole, AMB, or AME.

The incubation period was performed at 37°C in plas-
tic test tubes with horizontal rotation on a shaker at a rate of 120 rpm. Immediately after incubation, neu-

trophils were lysed by adding 9 ml of sterile distilled water to the reaction mixtures, serially diluting them
in 0.9% NaCl, and spreading them on Trypticase soy agar (BBL Microbiology Systems) plates to quantitate

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viable bacteria. Also, a fluorochrome microassay with acidine orange as an indicator of viable cellular material, described previously (20), was used to directly evaluate visual phagocytic and microbicidal functions of neutrophils exposed to the antifungal drugs. Blood was drawn in a syringe with no anticoagulant, and the appropriate concentration of ketoconazole, AMB, or AME was added and mixed. A leukocyte monolayer was prepared by dripping 0.2 ml of blood onto a sterile circular cover slip. All cover slips were incubated at 37°C in a humidified 5% CO2 incubator for 60 min. After incubation, the clot and serum were decanted and gently rinsed with Hank's balanced salt solution to remove nonadherent cells. A 0.1-ml amount of the standardized S. aureus 502A suspension was added to the monolayer which was placed on the cover slip (10 rpm) at 37°C for 90 min. The cover slips were then carefully rinsed and stained with 0.14% acidine orange for 45 s. The cover slips were mounted on glass slides, monolayer side down, and the edges were sealed with clear nail polish. Examination was performed with an ultraviolet fluorescent microscope under a 100x oil immersion objective. For the quantitation of phagocytic capacity, the total number of dead (red) and live (green) intracellular bacteria in 100 leukocytes was counted on each of three cover slips. Killing and phagocytic capacity were determined by the following formulas: % killing = (number of red organisms/number of red and green organisms per 100 leukocytes) x 100, and phagocytic capacity = (number of red and green organisms/100 leukocytes).

Statistics. Statistical analyses were made with Student's t test.

RESULTS

Cell viability was assessed by trypan blue dye exclusion. In vitro exposure of whole blood and washed neutrophils to concentrations ranging from 0 to 10 μg of ketoconazole per ml, 10 μg of AMB per ml, and 40 μg of AME per ml had no influence on cell death. Viability remained greater than 94% over a 3-h period.

Adherence of neutrophils in the presence of ketoconazole, AMB, and AME is summarized in Table 1. The general trend of all three antifungal agents on the ability of neutrophils to adhere to nylon wool was demonstrated, with decreased to normal adherence at low drug concentrations and increased adherence at high drug concentrations. The attainable therapeutic plasma levels of ketoconazole and AME demonstrated normal adherence at low concentrations and increased (P ≤ 0.01) adherence at higher concentrations. AMB decreased (P < 0.001) adherence at 1 μg/ml, but at 3 μg/ml, it approached normal adherence.

Data from five chemotaxis experiments (Fig. 1) of ketoconazole-treated cells responded normally, but the effects of both AMB and AME on migrating neutrophils under agarose were significantly depressed (P ≤ 0.001) at therapeutically attainable plasma concentrations (1 to 3 and 10 to 20 μg/ml, respectively). The chemotactic responses to 3 μg of AMB per ml and to 20 μg of AME per ml, those peak levels most likely to be obtained in plasma during therapy, were both 15% (P < 0.001) of the control.

Neutrophil chemiluminescence during phagocytosis was measured at 15 min and 2 h after cells were incubated with the antifungal agents. Preincubating the cells for 15 min did not alter the response compared with untreated cells, but exposing neutrophils for longer periods of time revealed an inhibitory response with AMB and AME, but not with ketoconazole (Fig. 2). Comparative data from five experiments suggest that

### Table 1. Effects of ketoconazole, AMB, and AME on neutrophil adherence

<table>
<thead>
<tr>
<th>Concen (μg/ml)</th>
<th>Ketoconazole</th>
<th>AMB</th>
<th>AME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of control</td>
<td>P value</td>
<td>% of control</td>
</tr>
<tr>
<td>1</td>
<td>(103)</td>
<td>NS</td>
<td>(86)</td>
</tr>
<tr>
<td>3</td>
<td>(117)</td>
<td>0.01</td>
<td>(100)</td>
</tr>
<tr>
<td>5</td>
<td>(123)</td>
<td>0.005</td>
<td>96</td>
</tr>
<tr>
<td>10</td>
<td>124</td>
<td>0.005</td>
<td>95</td>
</tr>
<tr>
<td>20</td>
<td>117</td>
<td>0.01</td>
<td>131</td>
</tr>
<tr>
<td>50</td>
<td>93</td>
<td>NS</td>
<td>155</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are therapeutic plasma levels.

** NS, Not significant.
AME is slightly more suppressive than AMB at therapeutic plasma levels.

Both bactericidal assays are summarized in Table 2. Bacterial destruction with ketoconazole-, AMB-, and AME-treated neutrophils was comparable to that with the untreated control cells by the colony count method. The fluorochrome microassay revealed that AMB-treated neutrophils had decreased phagocytic (P ≤ 0.005) and killing capacities (P = 0.01). Those treated with ketoconazole and AME were unchanged.

Four patients with mycotic infections were selected for in vivo antifungal studies, two treated with ketoconazole and two treated with AMB. Initial studies were performed before therapy. All patients were well into their course of therapy when blood was drawn, and the phlebotomy was performed to correspond to peak plasma levels of that particular drug.

Patient 1 (Table 3), a 1-year-old white male with chronic mucocutaneous candidiasis, was treated with ketoconazole. With increasing doses, chemotaxis and killing capacity (chemiluminescence) increased, and the previously increased neutrophil adherence was unaffected. Patient 2, a 33-year-old white female with helminthosporium sinusitis, was treated with ketoconazole. Chemotaxis was unchanged, and similar to patient 1, neutrophil adherence was unaffected but remained increased. Patient 3, 76 years old with aspergillosis, was treated with AMB and 5-fluorocytosine. Chemotaxis increased killing of the Aspergillus species increased, and adherence remained elevated. Patient 4, 12 years old with aspergillosis, was treated with AMB and 5-fluorocytosine. Chemotaxis was unchanged, killing of the Aspergillus species increased, and adherence remained elevated.

**DISCUSSION**

Several lines of clinical and experimental data suggest that mechanisms of cell-mediated immunity are most critical in protecting the host from systemic mycoses (24). However, disseminated fungal disease in patients with neutropenia (2) demonstrates the importance of phagocytic function. Amphotericin is presently the antifungal agent of choice for most disseminated fungal disease, although many investigators have suggested that AMB may be immunosuppressive (F. L. Delmonico and R. Rubin, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 16th, Chicago, Ill., abstr. no. 138).

**Table 2. Effects of ketoconazole, AMB, and AME on phagocytosis and intracellular killing of bacteria in normal blood phagocytes**

<table>
<thead>
<tr>
<th>Drug^a</th>
<th>Conc (μg/ml)</th>
<th>Colony count method (% killing)</th>
<th>Phagocytic capacity</th>
<th>% Killing^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>96</td>
<td>3.0</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>2 96</td>
<td>2.4</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 94</td>
<td>2.1</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 92</td>
<td>2.4</td>
<td>89^c</td>
<td></td>
</tr>
<tr>
<td>AMB</td>
<td>1 94</td>
<td>1.5^t</td>
<td>93</td>
<td>89^d</td>
</tr>
<tr>
<td></td>
<td>2 93</td>
<td>1.9^e</td>
<td>88^d</td>
<td>89^c</td>
</tr>
<tr>
<td></td>
<td>5 94</td>
<td>2.2</td>
<td>88^d</td>
<td></td>
</tr>
<tr>
<td>AME</td>
<td>10 92</td>
<td>3.3</td>
<td>97</td>
<td>94^g</td>
</tr>
<tr>
<td></td>
<td>15 95</td>
<td>3.2</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 94</td>
<td>2.9</td>
<td>94</td>
<td></td>
</tr>
</tbody>
</table>

^a S. aureus 502A was used in both assays.
^b Human phagocytes were incubated with drugs for 90 min.
^c Average number of intracellular bacteria per cell (100 leukocytes counted).
^d Number of red (dead) intracellular bacteria divided by the total number of intracellular bacteria in 100 leukocytes × 100.
^e P = 0.01.
^f P = 0.001.
^g P = 0.005.

The immune system is not routinely included in phase 1 studies of new drug toxicity. This is in part attributable not only to difficulty in standardization of current methodology, but perhaps also to a lack of full appreciation for the role that the immune mechanisms of the host play in many disease processes. It is now well accepted that full recovery from fungal disease requires some degree of host immune defense. Patients with poor immunological function are much more likely to succumb to severe fungal infections than those who are at least partially competent (24). It would therefore seem imperative that antifungal antibiotics not suppress host immune responses.

Recently, AME has been examined for clinical efficacy in the treatment of systemic mycoses. Experiences with patients appeared encouraging before a preliminary report of neuropsychiatric disturbances in patients who were receiving AME. Although these findings were supported by preliminary electroencephalographical and neuropathological data, they were mitigated by the presence of previous psychoses and fungal meningitis in some of the patients and by the administration of impure AME to a significant proportion of the affected individuals. Thus, although it is too soon to know whether this semisynthetic preparation will offer a therapeutic advantage or be too toxic for humans, manipulation of polyene antibiotic molecules still offers a means of improving the treatment of systemic mycoses. The newest oral broad-spectrum agent, ketoconazole, is active against many yeasts, dermatophytes, and dimorphous fungi (25) and has not as yet proven to be toxic in vivo. It was considered worthwhile, therefore, to assay the comparative effects on neutrophil function of the standard compound AMB, its semisynthetic analog AME, and its newest developed derivative, ketoconazole.

The present in vitro studies demonstrate no adverse immunotoxic effects of ketoconazole on neutrophil function, whereas AMB suppresses chemotactic responses, chemiluminescence, phagocytosis, and killing. At the same time, adherence was also decreased at low drug concentrations, whereas at higher concentrations, adherence was demonstrated to increase (Table 1). The mechanism for this selective effect of these antifungal drugs is intriguing. Evidence of the nature of membrane lesions resulting from polyene-sterol interactions supports the concept that the binding of polyenes to cell membranes induces changes in the physical properties of membranes (17). Further studies are in progress to delineate this phenomenon. The production of neutrophils was the only other aspect of neutrophil function not evaluated. However, other studies have noted an increase of greater than twofold in the absolute neutrophil count during day 1 of therapy with AMB and then a return to base-line counts by day 2 (12).

### Table 3. Patient studies before and during therapy with ketoconazole or AMB

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time in therapy</th>
<th>Drug</th>
<th>Chemotaxis*</th>
<th>Chemiluminescence (%)</th>
<th>Neutrophil adherence (%)</th>
<th>Absolute neutrophil count*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Migration</td>
<td>Random migration</td>
<td>Zymosan</td>
<td>Candida albicans</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>1,035 ± 123</td>
<td>669 ± 60</td>
<td>100</td>
</tr>
<tr>
<td>Patient</td>
<td>Before</td>
<td>Ketoconazole</td>
<td>692 ± 52</td>
<td>685 ± 19</td>
<td>89*</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>2 h post-50 mg</td>
<td></td>
<td>830 ± 60</td>
<td>627 ± 34</td>
<td>96</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>2 h post-100 mg</td>
<td></td>
<td>1,547 ± 267</td>
<td>544 ± 39</td>
<td>95</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Before</td>
<td>Ketoconazole</td>
<td>1,325 ± 92</td>
<td>783 ± 42</td>
<td>183</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>2 h post-200 mg</td>
<td></td>
<td>835 ± 42</td>
<td>458 ± 79</td>
<td>ND</td>
<td>ND*</td>
</tr>
<tr>
<td>3</td>
<td>Before</td>
<td>AMB</td>
<td>931 ± 124</td>
<td>416 ± 49</td>
<td>88</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>1 mg/kg</td>
<td></td>
<td>689 ± 148</td>
<td>165 ± 6</td>
<td>88</td>
<td>ND*</td>
</tr>
<tr>
<td>4</td>
<td>Before</td>
<td>AMB</td>
<td>403 ± 81</td>
<td>130 ± 20</td>
<td>71</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>1 mg/kg</td>
<td></td>
<td>745 ± 62</td>
<td>251 ± 3</td>
<td>107</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* Number of migrated cells in a single plane.
* Neutrophils per cubic millimeter.
* Percent of control.
* ND, Not determined.

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Chemotaxis represents an important augmenting mechanism by which leukocytes migrate into an area of inflammation. Patients with selective defects in chemotaxis are known to have recurrent bacterial, as well as fungal, infections (5). Phagocytosis and killing are, of course, the most critical steps in the final elimination of microbial organisms. Defects render the host susceptible to a wide spectrum of infectious processes. Suppression of these three mechanisms of immune function by AMB must be considered in evaluating therapeutic efficacy or in judging advantages of newer antifungal agents. In the case of AME, immunotoxic effects appear similar to those observed with AMB.

Patient data, although preliminary, did not suggest in vivo differences in the immunotoxic effects of ketoconazole as compared with AMB.

ACKNOWLEDGMENTS

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LITERATURE CITED