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Comparison of Ketoconazole and Amphotericin B in Interference with Thymidine Uptake by and Blastogenesis of Lymphocytes Stimulated with *Histoplasma capsulatum* Antigens

R. H. ALFORD[1,2]* AND B. B. CARTWRIGHT[1]

Veterans Administration Medical Center[1] and Vanderbilt University Medical School,[2] Nashville, Tennessee 37203

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Human peripheral blood lymphocytes stimulated with *Histoplasma capsulatum* yeasts were exposed in culture to graded concentrations of ketoconazole or amphotericin B and subsequently assessed for membrane integrity, thymidine uptake, and blastogenesis. Lymphocyte reactivity varied with concentration and duration of exposure to ketoconazole. Overt membrane toxicity resulted from exposure to 40 μg of ketoconazole per ml for 5 days, diminished thymidine uptake occurred with concentrations as low as 5 μg/ml, and 15 to 20 μg/ml caused a marked decrease in thymidine uptake and eventually diminished blastogenesis. The antilymphocyte action of ketoconazole was neutralized by increasing the concentration of human serum in cultures to 40% regardless of its cholesterol content. Amphotericin B activity was qualitatively similar but less pronounced.

Ketoconazole is toxic to slowly replicating mammalian cells such as fibroblasts (1) or macrophages (6) in culture only in concentrations greater than 100 μg/ml. This low degree of toxicity is presumably due in part to its minimal effect on mammalian cholesterol synthesis compared with its far greater effect on fungal ergosterol synthesis (17). Ketoconazole also has no demonstrable effect on the functions of peripheral human blood polymorphonuclear leukocytes in vitro (8). Recently, however, at concentrations as low as 5 μg/ml, ketoconazole has been found to retard replication of human granulocyte precursors (9) and impede thymidine uptake by human lymphocytes undergoing blastogenesis (R. H. Alford, Program Abstr. Conf. Antimicrob. Agents and Chemother. 22nd, Miami Beach, Fla., abstr. no. 489, 1982). We have further investigated the effects of ketoconazole on fungal antigen-stimulated thymidine uptake and blastogenesis of human lymphocytes, comparing its effects with those of amphotericin B.

MATERIALS AND METHODS

Lymphocyte cultures. Human venous peripheral blood mononuclear cells were separated by Ficoll-Hypaque cushioning, and 96 ± 2% pure lymphocytes were obtained by elution of nonadherent cells from Sephadex G-10 columns that had been incubated at 37°C, as previously described (2). Lymphocytes were virtually all initially viable, as defined by trypan blue exclusion. Lymphocyte suspensions contained 2 to 6% monocytes, occasional basophils, and some platelets. Two-milliliter cultures of 10^6 lymphocytes were established in loosely capped polypropylene tubes containing supplemented tissue culture medium (RPMI 1640; Flow Laboratories, Inc., Rockville, Md.) with 10% pooled normal human serum, as previously described (2); some experiments, however, specified 40% serum. Cultures were incubated in a 5% CO_2-supplemented humidified atmosphere for 5 days unless otherwise specified.

Antimycotics. Ketoconazole (a gift of Janssen Pharmaceutica, New Brunswick, N.J.) was initially dissolved in 0.1% HCl, subsequently diluted in RPMI 1640 medium or sodium desoxycholate-solubilized amphotericin B (E. R. Squibb & Sons, Princeton, N.J.), dissolved in water, and then diluted in RPMI 1640 medium. The drugs were then added in the indicated concentrations at the initiation of cultures unless otherwise specified. Antigens were either 10^4 heat-killed *H. capsulatum* cells (ATCC strain 12700) or, in some experiments, 8.5 μg of an autolysate prepared from yeast cells (13).

Thymidine uptake. During the last 4 h of incubation, thymidine uptake was detected by pulsing with 2.0 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.; specific activity, 52.4 Ci/mM) as previously described (2). Cultures were thoroughly washed three times with serum containing supplemented RPMI 1640 medium before the addition of tritiated thymidine to remove ketoconazole, amphotericin B, or any accumulated "cold" thymidine which could otherwise be present during the uptake period. Results were expressed in disintegrations per minute. Cultures were run in duplicate, and experiments were repeated a minimum of three times on separate occasions with different histoplasmin skin test-positive cell donors.

Enumeration of blasts. Blast forms of lymphocytes

were enumerated microscopically by examining Wright-stained smears of 500 cells.

Cholesterol adsorption. Cholesterol and lipoproteins were removed by a single adsorption of pooled human serum with Cabosil (Packard Instruments, Downers Grove, Ill.; 4 g/200 ml of serum) that was stirred at 4°C overnight. Cholesterol in serum was quantitated enzymatically at 37°C (Biodynamics/bmc, Indianapolis, Ind.).

RESULTS

Ketoconazole in concentrations of 40 μg/ml and higher caused disruption of the integrity of lymphocyte plasma membranes, as indicated by the failure of many lymphocytes to exclude trypan blue. Inhibition of thymidine uptake was evident in 10% of the serum-containing cultures of some lymphocytes in concentrations as low as 5 μg/ml (Fig. 1). Statistically significant inhibition of thymidine uptake occurred with cells from all donors at 15 and 20 μg/ml. Comparable results occurred with either the particulate (whole-cell) or soluble (autolysate) antigen (data not shown). Inhibition of thymidine uptake also resulted from incorporation of amphotericin B in cultures (Fig. 2). Its effect was somewhat less than that of ketoconazole; it was statistically significant only at 20 μg/ml. Inhibition of thymidine uptake by 20 μg of ketoconazole per ml or amphotericin B in 5-day cultures was also associated with impairment of blastogenesis (Table 1). At lower concentrations of antimycotics, enumeration of blasts was too insensitive for comparison with thymidine uptake.

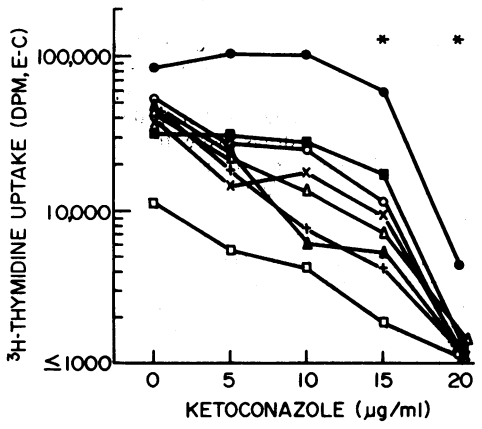

FIG. 1. Dose-related suppression of thymidine uptake by *H. capsulatum*-stimulated lymphocytes. Each symbol represents results from one of eight different histoplasmin skin test-positive cell donors. E−C, Experimental minus control (unstimulated) values. Asterisks (*) indicate statistical difference by a *t* test for independent means of pooled data from that of controls ($P < 0.01$).

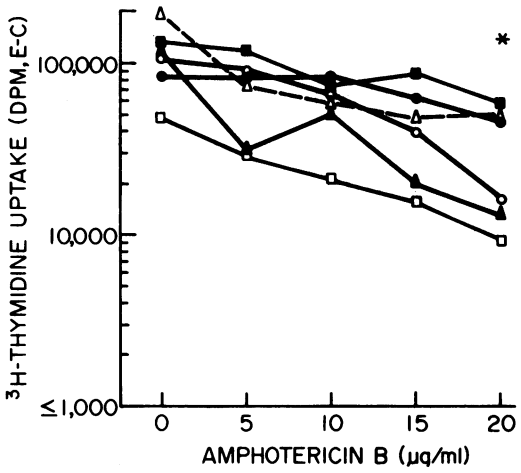

FIG. 2. Dose-related suppression of thymidine uptake by *H. capsulatum*-stimulated lymphocytes. Each symbol represents results from one of six different cell donors. E−C, Experimental minus control (unstimulated) values. The asterisk (*) indicates the statistical difference from controls ($P < 0.01$).

The effect of human serum on ketoconazole-induced inhibition of thymidine uptake is shown in Fig. 3. At all concentrations of ketoconazole tested, 40% serum allowed increased thymidine uptake, although the effect was statistically significant only at 20 μg/ml. To evaluate possible roles for cholesterol on the neutralization by serum of ketoconazole's inhibitory effect on thymidine uptake by lymphocytes, paired cultures in which native or Cabosil-treated pooled serum was incorporated were compared (Fig. 3). Despite the removal of 99% of its cholesterol, the serum still neutralized ketoconazole's ability to inhibit thymidine uptake by lymphocytes.

TABLE 1. Comparison of [^{3}H]thymidine uptake and blastogenesis of antimycotic-treated lymphocytes

Antimycotic[a]	Duration of antimycotic exposure		Thymidine uptake (E − C ± SD)[b]	Lymphoblasts (% ± SD)[c]
	5 days	30 min		
None	−	−	42,961 ± 30,933	4.8 ± 2.6
Ketoconazole	+	−	7,229 ± 3,606	1.6 ± 0.8
	−	+	5,062 ± 3,088	4.3 ± 1.8
Amphotericin B	+	−	18,203 ± 8,317	1.5 ± 0.2
	−	+	51,728 ± 42,847	3.3 ± 0.8

[a] Ketoconazole and amphotericin B were both used at a concentration of 20 μg/ml.

[b] Three cell donors, each run in duplicate. E − C, Experimental minus control with unstimulated culture with [^{3}H]thymidine uptake of 1,571 ± 379 dpm.

[c] The control with unstimulated cultures contained 0.8 ± 0.4% blasts.

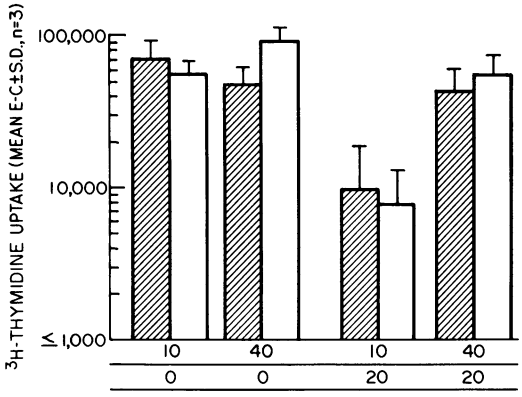

FIG. 3. Neutralizing action of 40% human serum on thymidine uptake by lymphocytes compared with that of 10% serum. Cholesterol-depleted serum (□, 3 mg of cholesterol per ml) had the same effect as native pooled serum (▨, 238 mg/dl). Vertical bars indicate standard deviation; three cell donors. On the abscissa, the numbers on the top row indicate the percentage of serum; those on the bottom row indicate the concentration of ketoconazole (in micrograms per milliliter).

To ascertain something about the rates at which ketoconazole or amphotericin B affected thymidine uptake, 20 μg of either drug per ml was added at various times to cultures begun simultaneously as little as 10 min before washing for thymidine uptake (Fig. 4). Ketoconazole was equally effective in impairing thymidine uptake at all intervals tested, although it definitely affected blastogenesis only when it was incorporated for the full 5 days. Amphotericin B also promptly inhibited thymidine uptake but was less potent on a weight-for-weight basis.

DISCUSSION

Clotrimazole, another *N*-substituted imidazole antimycotic, suppresses thymidine uptake by human lymphocytes in culture (14) at concentrations of 2 to 10 μg/ml. Amphotericin B is somewhat less active in that regard, and its solubulizer, sodium desoxycholate, is inactive (14). The antilymphocyte actions of both of these antimycotics can be neutralized by the addition of 45% autologous serum (14).

In our studies, thymidine uptake by lymphocytes in cultures with 10% pooled human serum was usually impaired by ketoconazole in concentrations as low as 5 μg/ml (10^{-5} M). In concentrations of 15 to 20 μg/ml (4×10^{-5} M), ketoconazole significantly impaired both thymidine uptake and blastogenesis without overt membrane damage. In concentrations of ≥ 40 μg/ml (10^{-4} M), ketoconazole caused the loss of membrane integrity of many lymphocytes cultured for 5 days, as judged by their failure to exclude trypan blue.

Antimycotic imidazoles exhibit a wide array of biological activities. They can affect sterol and lipid synthesis (12, 18), membrane-bound enzymes (16), oxidative enzymatic processes (10), and thromboxane (15). The specific action that causes inhibition of thymidine uptake by antigen-stimulated lymphocytes is unknown. The diverse, concentration-dependent effects of antimycotic imidazoles on sterol-containing membranes have been extensively studied (3, 12, 18). In low concentrations, inhibition of sterol synthesis occurs, whereas in higher concentrations, direct membrane damage is produced that is much less pronounced with ketoconazole than with clotrimazole or miconazole (12). Liposomes are also much more susceptible to disruption by high concentrations of miconazole than of ketoconazole (12, 16). Antimycotic imidazole-mediated alterations in the membrane composition of sterols, fatty acids, and phospholipids appear to occur gradually (3), and secondary effects on membrane-bound enzymes such as cytochrome oxidase also require hours to become apparent (3).

The rapid rate at which ketoconazole suppressed thymidine uptake by stimulated lymphocytes may be indicative of its mechanism of action in this regard. This extremely rapid effect was evaluated in light of a recent suggestion that readily reversible inhibition of thymidine uptake by stimulated lymphocytes could result from small-molecular-weight lipophilic compounds that readily enter and non-specifically alter bilayer membrane function without appreciably affecting DNA synthesis or blastogenesis (11). In our experiments, lipophilic (4) ketoconazole action differed in that its effect on [^{3}H]thymidine uptake, though extremely rapid in its onset,

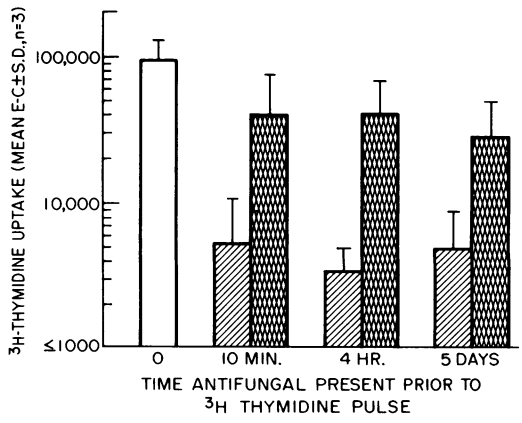

FIG. 4. Prompt effect of antifungal agents in inhibiting thymidine uptake by *H. capsulatum* antigen-stimulated lymphocytes. ▨, 20 μg of ketoconazole per ml; ▩, 20 μg of amphotericin B per ml. (Vertical bars indicate standard deviation; three cell donors).

persisted after drug-containing supernatant fluids were thoroughly removed. Further, we verified morphologically the impairment of blastogenesis after lymphocytes had been cultured for 5 days with ketoconazole (Table 1). Amphotericin B also affected thymidine uptake by lymphocytes promptly, but to a lesser degree, possibly because of its larger molecular size or more avid protein binding that retarded access to critical membrane sites.

Low concentrations of miconazole very rapidly inhibit membrane-bound ATPase (7) in yeast cells. It is possible that ketoconazole promptly affects lymphocyte membrane-bound thymidine kinase, thereby inhibiting phosphorylation of thymidine via the thymidine rescue pathway.

Thus, ketoconazole appears to affect lymphocytes through the use of several mechanisms, depending on its concentration and duration of exposure. High concentrations of ketoconazole that can be achieved therapeutically with large doses (5) antagonized thymidine uptake by antigen-stimulated human lymphocytes rapidly, interfered with lymphocyte blastogenesis with longer exposure, and in concentrations greater than those used clinically, caused overt membrane damage. The antilymphocytic actions were neutralized by human serum. The influence of ketoconazole on antigen-mediated lymphocyte function could have important overall as well as clinical implications.

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