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Inhibition by Ketoconazole of Mitogen-Induced DNA Synthesis and Cholesterol Biosynthesis in Lymphocytes

THOMAS M. BUTTKE[1]* AND STANLEY W. CHAPMAN[2,3]

Departments of Microbiology[1] and Medicine,[2] University of Mississippi Medical Center, and Medical Service, Veterans Administration Medical Center,[3] Jackson, Mississippi 39216

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The effects of ketoconazole on mitogen-induced DNA synthesis and cholesterol biosynthesis in human and murine lymphocytes have been examined. Ketoconazole concentrations which do not affect cell viability (0.1 to 10 μg/ml) in culture led to a dose-dependent inhibition of DNA synthesis, as measured by [^{3}H]thymidine incorporation, induced by either T-cell or B-cell mitogens. At drug concentrations 5- to 10-fold lower, ketoconazole inhibited the incorporation of [^{14}C]acetate into cholesterol, with a resultant accumulation of [^{14}C]lanosterol. The suppressive effects of ketoconazole on DNA synthesis were reversed by increasing the concentration of human serum in the culture medium from 5 to 20%. The depletion of lipoproteins in human serum by density centrifugation reduced the cholesterol content by 90% but did not affect the ability of the serum to overcome the inhibition by ketoconazole of DNA synthesis. Unlike DNA synthesis, cholesterol biosynthesis was not restored by 20% fresh human serum or lipoprotein-depleted human serum. These results demonstrate that ketoconazole potently inhibits DNA synthesis and cholesterol synthesis in mitogen-stimulated lymphocytes at drug concentrations obtained therapeutically. Further, the uncoupling of endogenous cholesterol synthesis and DNA synthesis indicates at least two levels of action of ketoconazole in mammalian lymphocytes.

Ketoconazole is an imidazole antifungal agent with potent activity against various superficial and systemic mycoses (17). The precise mode of action of ketoconazole is unknown, but the imidazoles generally seem to inhibit the growth of fungi by increasing plasma membrane permeability (2). Increased permeability may result from the direct insertion of the imidazoles into the membrane bilayer or from the action of the imidazoles on fungal lipid synthesis (2, 3). The latter is consistent with the findings that miconzaole, clotrimazole, and ketoconazole inhibit ergosterol synthesis in fungi by blocking the demethylation of lanosterol (18, 24, 25). As a result, fungi accumulate various alkylated precursor sterols which may subsequently replace ergosterol as the predominant membrane sterol. Thus, the fungistatic effect of the imidazoles may result from their inhibitory action on ergosterol biosynthesis.

Two of the imidazoles, miconazole and ketoconazole, have also been shown to block lanosterol demethylation in animal cells, resulting in an inhibition of cholesterol biosynthesis (19, 24, 25). Since lanosterol cannot satisfy the sterol requirement of animal cells in culture (5, 15), it is surprising that the imidazoles display such a selective toxicity for fungi in vivo. Van den Bossche et al. have suggested that this selective toxicity stems from the ability of animal cells to satisfy their sterol requirements by incorporating exogenous cholesterol from serum (23). However, some mammalian cells, such as lymphocytes, appear to require a period of endogenous cholesterol synthesis before proliferation (1, 6–8, 10, 20). Such cells may be sensitive to the inhibition of cholesterol synthesis by imidazoles, with resultant cell dysfunction or toxicity. To test this possibility, we examined the effects of ketoconazole on DNA synthesis and cholesterol synthesis in mitogen-stimulated human and mouse lymphocytes. Our results show that ketoconazole does inhibit both synthetic processes, but the inhibition of DNA synthesis is not directly related to the effects of ketoconazole on cholesterol biosynthesis.

MATERIALS AND METHODS

Human subjects. Control blood donors were obtained from healthy laboratory personnel and medical staff at the University of Mississippi Medical Center and the Veterans Administration Medical Center, Jackson, Miss. Informed consent was obtained from all of the donors in this study, and the protocol was approved by the Institutional Review Board of the University of Mississippi Medical Center.

Mice. Male and female BALB/c mice, 8 to 12 weeks of age, were obtained from a colony maintained at the University of Mississippi Medical Center.

In vitro culture conditions. Human peripheral blood mononuclear cells were isolated from heparinized blood by gradient centrifugation on Ficoll-Hypaque (Mono-Poly Resolving Medium; Flow Laboratories, Inc., McLean, Va.). Cells were washed three times in RPMI 1640 (Flow) and suspended in RPMI 1640 containing glutamine (300 mg/liter), penicillin (50 U/ml), streptomycin (50 μg/ml), and the desired concentrations of the different protein supplements.

Mice were sacrificed by cervical dislocation, and the splenic cell suspensions were depleted of erythrocytes and macrophages as described previously (4). In some cases, the mixed lymphocytes were further separated into B-cell and T-cell populations by positive selection techniques (4). Isolated cells were suspended in RPMI 1640 containing 0.2% $NaHCO_3$, penicillin (50 U/ml), streptomycin (50 μg/ml), and the desired protein supplements.

Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 (human cells) or 7% CO_2 (murine cells). Cell viabilities were determined by trypan blue exclusion.

Protein supplements for in vitro cultures. Autologous human serum (AHS) was obtained fresh from each cell donor on the day lymphocytes were isolated. Lipoprotein-free human serum was prepared by adding solid KBr to raise the density of the serum to 1.215 g/ml, followed by centrifugation at 120,000 × *g* for 20 h at 15°C. After the lipoprotein layer was removed, the lipoprotein-free serum was dialyzed against three changes of Dulbecco phosphate-buffered saline (11). Mock human serum was prepared identically to lipoprotein-free serum except that the lipoprotein band was not removed before dialysis. Fetal bovine serum (FBS; Flow) was heat inactivated at 56°C for 60 min and stored frozen. Human serum albumin (HSA) was obtained as a 25% solution (Cutter Laboratories, Berkeley, Calif.) and stored at room temperature. Serum cholesterol concentrations were measured with a cholesterol oxidase kit (Sigma Chemical Co., St. Louis, Mo.).

Proliferation assays. Lymphocytes were cultured in 96-well microtiter plates at a concentration of 2×10^5 human cells or 5×10^5 mouse cells per 0.2 ml of culture. Human lymphocytes were incubated with phytohemagglutinin P (PHA; Difco Laboratories, Detroit, Mich.) at 10 μg per well or the formaldehyde-fixed Cowan I strain of *Staphylococcus aureus* at 10^8 bacteria per well to stimulate T lymphocytes or B lymphocytes, respectively. *S. aureus* Cowan I, kindly provided by Arthur White (Indiana University, Indianapolis), was prepared by the method of Schuurman et al. (21). The optimal PHA responses were observed after 3 days in culture, whereas the maximum *S. aureus* Cowan I response occurred after 5 days in culture. Murine lymphocytes were stimulated with the T-cell mitogen PHA at 1 μg per well in a 2-day incubation or the B-cell mitogen lipopolysaccharide (LPS; *Salmonella typhimurium*, Westphal; Difco) at 10 μg per well in a 3-day incubation.

The effects of ketoconazole on mitogen-induced proliferation were determined by adding various concentrations of the drug to the cell cultures at the time of mitogen addition. Ketoconazole (Janssen Pharmaceuticals, New Brunswick, N.J.) was initially solubilized in 0.5 N HCl to yield a 2-mg/ml solution, followed by a 1:10 dilution with RPMI 1640. Further dilutions were made by using 0.5 N HCl–RPMI 1640 (1:9, vol/vol), with this same diluent being added to control cells.

Mitogen-induced DNA synthesis was assayed by labeling cells, in triplicate or quadruplicate, with 0.5 μCi of [*methyl*-^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) during the final 16 to 18 h of incubation. The cells were subsequently harvested onto glass fiber filters with a multichannel automated harvester (Brandel Biomedical, Gaithersburg, Md.), and the amount of [^{3}H]thymidine incorporated was measured by liquid scintillation counting. The mean counts per minute and standard deviation were calculated for each culture condition. In some cases, the data are presented as the percentage of the control, where % of control = (counts per minute of mitogen-stimulated cultures with ketoconazole × 100)/(counts per minute of mitogen-stimulated cultures with diluent).

Lipid biosynthesis. Sterol synthesis was assayed by measuring the incorporation of [1-^{14}C]acetate (New England Nuclear). Human (5×10^6) or mouse (1×10^7) lymphocytes were cultured in 2 ml of medium in 24-well cultures plates containing the optimum dose of mitogen and various concentrations of ketoconazole. After 24 h of incubation, 10 μCi of [^{14}C]acetate was added to each well, and the cultures were incubated for an additional 6 h. The labeled cells were collected by centrifugation, washed twice with RPMI 1640, and saponified with ethanolic KOH at 70°C for 1 h. Nonsaponifiable lipids were extracted into petroleum

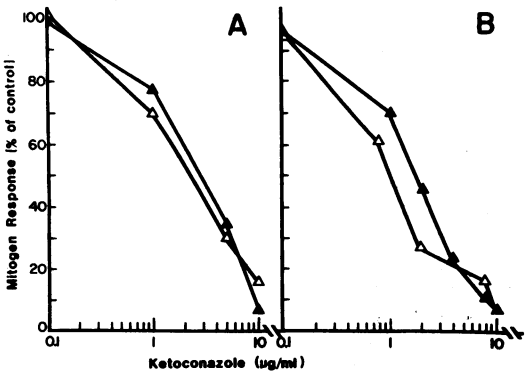

FIG. 1. Inhibition by ketoconazole of DNA synthesis in human lymphocytes. Human lymphocytes were cultured in the presence of 5% AHS (▲) or 1% HSA (△) and stimulated with the T-cell mitogen PHA (A) or the B-cell mitogen *S. aureus* Cowan I (B). Various concentrations of ketoconazole were added at the time of mitogen addition. Data shown are from a representative experiment. In the absence of ketoconazole, the amounts of [^{3}H]thymidine incorporated (counts per minute) were as follows: PHA-stimulated cells in the presence of 5% AHS, 151,862; PHA-stimulated cells in the presence of 1% HSA, 58,956; *S. aureus* Cowan I-stimulated cells in the presence of 5% AHS, 19,467; *S. aureus* Cowan I-stimulated cells in the presence of 1% HSA, 51,513.

ether-diethyl ether (1:1, vol/vol) and separated into C-4 desmethyl sterols (primarily cholesterol), C-4 monomethyl sterols, and C-4 dimethyl sterols by thin-layer chromatography on silica gel plates developed with chloroform. To account for procedural losses, [7-^{3}H(N)]cholesterol (5×10^5 cpm; New England Nuclear) was added to each lipid sample as an internal standard. Radioactive sterols were located by autoradiography and quantified by liquid scintillation counting.

The ^{14}C-labeled sterol which accumulated in the ketoconazole-treated cells was identified as lanosterol based upon its mobility in several chromatographic systems. Non-saponifiable lipids were fractionated into sterols and nonsterols by high-pressure liquid chromatography with a Perkin-Elmer series 3 liquid chromatograph equipped with a Supelcosil LC-18 reversed-phase column (0.46 by 25 cm; Supelco, Bellefonte, Pa.). Lipids were eluted with methanol-water, using a linear gradient from methanol-water, 5:95 (vol/vol) to methanol-water, 99:1 (vol/vol). The flow rate was 2 ml/min for 30 min, and 2-ml fractions were collected. Fractions corresponding to sterols (fractions 15 to 22) were collected and further analyzed by thin-layer chromatography on silica gel plates developed with 3% ethyl acetate in methylene chloride. The C-4 dimethyl sterol fraction was eluted, and the recovered sterols were further analyzed by argentation thin-layer chromatography on silver nitrate-impregnated plates developed with 3% ethyl acetate in methylene chloride.

RESULTS

Effects of ketoconazole on lymphocyte proliferation. The effect of increasing concentrations of ketoconazole on the mitogenic response of human lymphocytes cultured in the presence of 5% AHS is shown in Fig. 1. In the absence of ketoconazole, T lymphocytes were stimulated by PHA, whereas B lymphocytes responded to *S. aureus* Cowan I. The addition of ketoconazole resulted in a dose-dependent reduction of [^{3}H]thymidine incorporation in response to both mitogens, indicating an inhibition of mitogen-induced proliferation. Low concentrations of the drug (0.1 μg/ml) had no effect, but at 10 μg/ml, DNA synthesis was inhibited by 80 to 90%. In 11 donors, the mean concentration of ketoconazole which caused 50% inhibition (I_{50}) of PHA-induced DNA synthesis was 5.4 (±1.5) μg/ml, whereas for *S. aureus* Cowan I-induced DNA synthesis, the I_{50} in 4 donors was 3.5 (±1.6) μg/ml. Cells cultured in the presence of 1% HSA were consistently found to be slightly more susceptible to ketoconazole than were AHS-grown cells. In media supplemented with HSA, the I_{50} in 12 donors for PHA-induced DNA synthesis was 2.5 (±1.2) μg/ml, and for *S. aureus* Cowan I-induced DNA synthesis, the I_{50} in 4 donors was 1.3 (±0.2) μg/ml. Based upon results obtained with PHA and *S. aureus* Cowan I it appears that T cells and B cells are equally susceptible to the inhibitory effects of ketoconazole on DNA synthesis. In agreement with an earlier study (9), we found that concentrations of ketoconazole below 100 μg/ml did not affect cell viability (data not shown). Thus, at drug concentrations which are readily achieved therapeutically, ketoconazole potently inhibits mitogen-induced human lymphocyte proliferation in vitro.

Additional experiments were performed to examine the effects of ketoconazole on murine lymphocyte proliferation. The stimulation of T and B mouse lymphocytes was also inhibited by ketoconazole (Fig. 2). Again, T and B lymphocytes were equally susceptible, whether mixed lymphocytes or pure T-cell or B-cell populations were examined. A comparison of the data shown in Fig. 1 and 2 suggests that murine lymphocytes are more susceptible to ketoconazole inhibition (I_{50} = 1 μg/ml) than are human lymphocytes. However, this difference is probably a reflection of the different serum supplements used; murine lymphocytes were routinely cultured in 5% FBS, whereas human lymphocytes were cultured in 5% AHS or 1% HSA. We later found (see below) that FBS-cultured human lymphocytes were more susceptible to ketoconazole than were AHS-cultured human lymphocytes.

In subsequent experiments, it was found that increasing or decreasing the doses of mitogens did not overcome the inhibitory effects of keto-

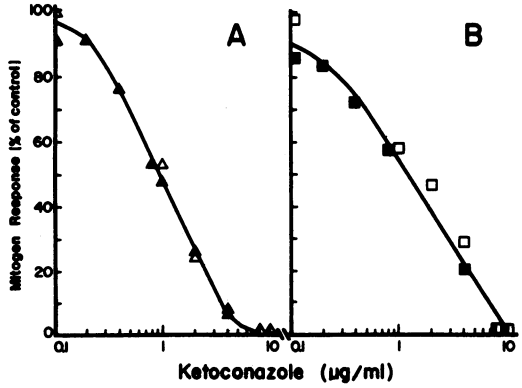

FIG. 2. Inhibition by ketoconazole of DNA synthesis in murine lymphocytes. Mixed lymphocyte populations (▲, ■) and purified populations of T cells (△) and B cells (□) were cultured in the presence of 5% FBS and stimulated with PHA (A) or LPS (B). Various concentrations of ketoconazole were added at the time of mitogen addition. Data shown are from a representative experiment. In the absence of ketoconazole, the amounts of [^{3}H]thymidine incorporated (counts per minute) were as follows: mixed lymphocytes with PHA, 76,641; mixed lymphocytes with LPS, 87,757; T cells with PHA, 132,363; B cells with LPS, 43,727.

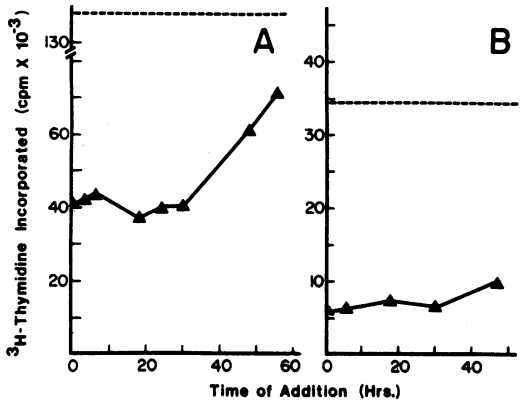

FIG. 3. Effect of delayed addition of ketoconazole on mitogen-induced DNA synthesis. Human lymphocytes (A) were cultured in the presence of 5% AHS and 10 μg of PHA per well. At various times after mitogen addition, cultures received 10 μg of ketoconazole per ml (▲). Murine lymphocytes (B) were cultured in the presence of 5% FBS and 10 μg of LPS per well. At various times, cultures received 4 μg of ketoconazole per ml (▲). Cell cultures were labeled with [^{3}H]thymidine between 56 and 72 h after the addition. The amounts of [^{3}H]thymidine incorporated in the absence of ketoconazole are indicated by the dashed line. Data shown are the means of quadruplicate (A) or triplicate (B) cultures from representative experiments. Standard deviations were within 7% of the means.

conazole and that ketoconazole suppressed DNA synthesis in human and mouse lymphocytes without altering the time course of the mitogenic response (data not shown). We also found that ketoconazole could be added as late as 50 to 55 h after mitogen addition and still inhibit DNA synthesis (Fig. 3). Thus, ketoconazole does not inhibit DNA synthesis merely by interfering with an early step in the activation process.

Effects of ketoconazole on cholesterol biosynthesis. Since ketoconazole has been shown to block lanosterol demethylation in animal cells (5, 7, 8), it was of interest to examine cholesterol synthesis in the ketoconazole-treated cells in vitro. In the absence of ketoconazole, human and mouse lymphocytes incorporated [^{14}C]acetate into cholesterol and C-4 monomethyl and C-4 dimethyl sterols (Fig. 4). Cholesterol accounted for 95 to 97% of the sterols labeled in murine lymphocytes, but in human lymphocytes, the percentage of label in cholesterol ranged from 50 to 70, with the balance being primarily lanosterol. Adding ketoconazole to the culture medium resulted in a decreased amount of label being incorporated into cholesterol with a concomitant increase in the amount of [^{14}C]acetate incorporated into C-4 dimethyl sterols (Fig. 4). Further analysis of the sterol accumulating in the drug-treated mouse cells showed that 92% of the label was associated with lanosterol, whereas 8% was identified as 24,25-dihydrolanosterol. The lack of accumulation of C-4 monomethyl sterols implies that ketoconazole specifically blocked the removal of the C-14 methyl group and thus prevented the normal oxidative demethylation of lanosterol in the drug-treated lymphocytes.

In both human and mouse lymphocytes, the addition of 1.0 μg of ketoconazole per ml led to a 30% inhibition of total sterol synthesis, whereas cholesterol synthesis was inhibited by 80 to 90% (Fig. 5). Similar results were obtained when cells were stimulated with T-cell or B-cell mitogens or when human lymphocytes were cultured in the presence of 1% HSA (data not shown). Interestingly, in both human and mouse cell systems, the concentration of ketoconazole needed for I_{50} of cholesterol biosynthesis (0.2 to 0.3 μg/ml) was 5- to 10-fold lower than the level of drug needed to obtain a similar inhibition of DNA synthesis.

Rescue of ketoconazole inhibition of DNA synthesis by human serum. In previous studies of the effects of various antibiotics on lymphocyte proliferation, it was reported that the degree of inhibition observed was dependent upon the concentration of serum present in the culture medium (14). Therefore, we evaluated the different protein supplements used in the present study for their ability to overcome the inhibitory effects of ketoconazole on DNA synthesis. Figure 6 shows that in the absence of drug, human cells cultured in the presence of 5% AHS, 5% FBS, or 1% HSA responded equally well to PHA. Raising the concentrations of sera to 20% had no effect on lymphocyte proliferation,

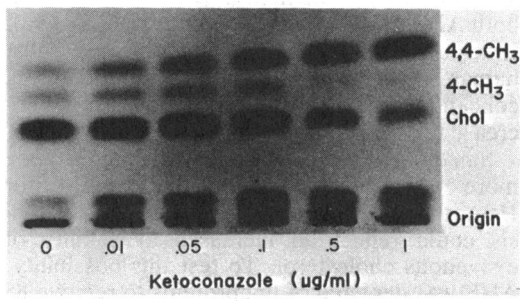

FIG. 4. Autoradiogram of thin-layer chromatography separation of ^{14}C-labeled non-saponifiable lipids recovered from human lymphocytes. Lymphocytes were cultured in medium containing 5% AHS, 10 μg of PHA per well and various concentrations of ketoconazole. Cells were labeled with [^{14}C]acetate, and lipids were analyzed as described in the text. Chol, C-4 desmethyl sterols (primarily cholesterol); 4-CH$_3$, C-4 monomethyl sterols; 4,4-CH$_3$, C-4 dimethyl sterol (primarily lanosterol).

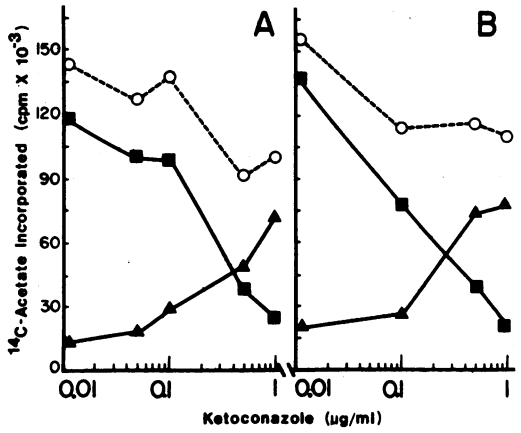

FIG. 5. Effect of ketoconazole on sterol synthesis in PHA-induced lymphocytes. Human lymphocytes (A) and murine lymphocytes (B) were cultured in the presence of 5% AHS and 5% FBS, respectively. Sterols were labeled with [^{14}C]acetate and analyzed as described in the text. Symbols: ○, total sterols; ■, cholesterol; ▲, lanosterol. Data are the results of representative experiments.

whereas increasing the level of HSA decreased the mitogenic response. The addition of 10 µg of ketoconazole per ml to human cells cultured in 5% AHS, 5% FBS, or 1% HSA caused a 90 to 95% inhibition of mitogen-induced DNA synthesis. However, increasing the concentration of AHS to 20% almost completely (77%) reversed the inhibition observed in the ketoconazole-treated cells. Due to the enhanced susceptibility of FBS-supplemented human cells to ketoconazole, high concentrations of FBS were less effective in restoring DNA synthesis. Similar results were obtained with mouse lymphocytes when both AHS and FBS were used (data not shown). In contrast to the serum-supplemented cells, human lymphocytes cultured with increasing concentrations of HSA did not display an increased resistance to ketoconazole (Fig. 6).

Since AHS would be expected to provide more exogenous cholesterol than would FBS or HSA, the ability of AHS to rescue DNA synthesis could reflect an increased availability of exogenous cholesterol. To test this possibility, AHS was depleted of lipoproteins to remove 85 to 90% of the cholesterol, and the ability of the lipoprotein-free serum to restore DNA synthesis was examined. Figure 7 compares the abilities of AHS, mock lipoprotein-free human serum, lipoprotein-free human serum, and FBS to rescue mitogen-induced proliferation as a function of the cholesterol content of the sera. From the graph it is obvious that there was no correlation between the cholesterol content and the ability to rescue DNA synthesis. For example, cultures supplemented with 20% lipoprotein-free serum contained only 28 µg of cholesterol per ml, yet the recovery of DNA synthesis was equal to the recoveries observed with 20% mock lipoprotein-free serum or 15% AHS, each of which provided 170 µg of cholesterol per ml. Similarly, the inability of FBS to rescue DNA synthesis was not attributable to low cholesterol content since the level of cholesterol in cultures supplemented with 20% FBS was actually higher (55 µg/ml) than the levels obtained with 20% lipoprotein-free human serum. It therefore appears that some component(s) of human serum is able to overcome the inhibition of DNA synthesis by ketoconazole, and this component(s) either is not present or is present in low amounts in FBS. Our results also show that the unidentified component(s) is probably not cholesterol or albumin.

Effects of increasing AHS levels on cholesterol synthesis in ketoconazole-treated cells. The recovery of DNA synthesis observed in ketoconazole-treated cells supplemented with 20% AHS could reflect an inactivation of the drug by AHS. If this were the case, cholesterol synthesis in ketoconazole-treated cells should also have been restored by 20% AHS. However, as shown in Fig. 8, this was not the case. In the example shown, increasing the content of AHS from 5 to

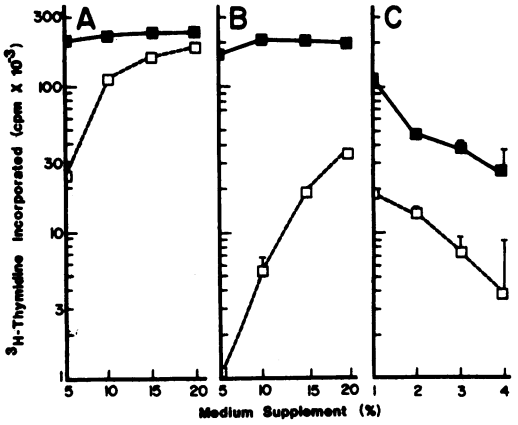

FIG. 6. Influence of medium supplements on the inhibition by ketoconazole of PHA-induced DNA synthesis. Human lymphocytes were cultured in medium containing the indicated concentrations of (A) AHS, (B) FBS, and (C) HSA in the absence (■) or presence (□) of 10 µg of ketoconazole per ml. Data are the means of quadruplicate cultures from representative experiments. In the presence of 20% AHS, the amounts of [^{3}H]thymidine incorporated were 238,107 cpm in the absence of ketoconazole and 184,711 cpm in the presence of 10 µg of ketoconazole per ml. Except where indicated by error bars, standard deviations were within 8% of the means.

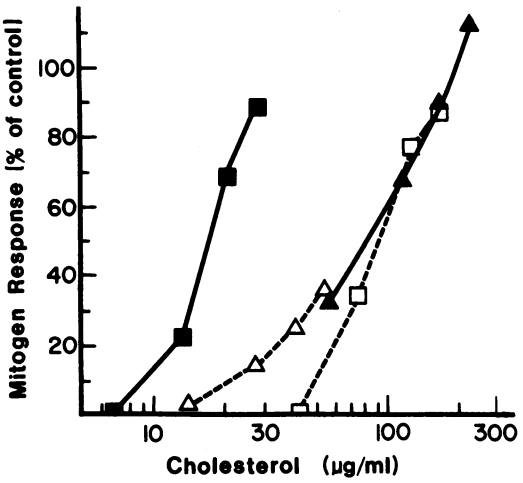

FIG. 7. Cholesterol content of serum supplements used to restore DNA synthesis in ketoconazole-treated cells. In each case, human lymphocytes were cultured in medium containing 5, 10, 15, or 20% AHS (▲), mock lipoprotein-free human serum (□), lipoprotein-free human serum (■), or FBS (△), and PHA-induced DNA synthesis was measured in the presence and absence of 10 μg of ketoconazole per ml. Cholesterol concentrations of the various serum supplements were determined enzymatically as described in the text. Data shown represent the amount of [^{3}H]thymidine incorporated by ketoconazole-treated cells relative to that incorporated by the corresponding control cells.

20% led to a decline in total sterol synthesis, with cholesterol biosynthesis being somewhat more susceptible to AHS inhibition than was lanosterol biosynthesis. In ketoconazole-treated cells, raising the AHS content in the medium to 20% led to a similar decrease in total sterol synthesis, but lanosterol still accounted for more than 90% of the sterols being synthesized, regardless of the AHS concentration. Identical results were obtained with human and mouse lymphocytes cultured with mock lipoprotein-free human serum or lipoprotein-free human serum (data not shown). Since increasing the AHS content did not restore cholesterol biosynthesis, it is unlikely that the recovery of DNA synthesis in 20% AHS-supplemented cells merely reflects an inactivation of the ketoconazole by AHS. These results also demonstrate that DNA synthesis in mitogen-stimulated lymphocytes does not require an early period of endogenous cholesterol biosynthesis.

DISCUSSION

The data presented in this study show that ketoconazole, at drug concentrations readily achieved in vivo when treating fungal disease, inhibits DNA synthesis and cholesterol synthesis in mitogen-stimulated lymphocytes. The level of ketoconazole which inhibited DNA synthesis by 90 to 95% was ca. 1/10 of the amount needed to reduce cell viability, implying a specific effect of ketoconazole on lymphocyte proliferation. Previous workers have obtained similar results with clotrimazole (12), and we recently found that miconazole can also block DNA synthesis in lymphocytes without affecting cell viability (unpublished data). Further studies are necessary to determine whether each of the imidazoles interferes with cell proliferation via a common mechanism. Although our findings and the results of others (12) suggest that the imidazoles may be potent immunosuppressants, it is important to note that for ketoconazole, at least, the inhibition of DNA synthesis was dependent upon the cell culture conditions employed. Lymphocytes cultured in the presence of either HSA, FBS, or 5% AHS were extremely sensitive to 10 μg of ketoconazole per ml, whereas cells cultured in 20% AHS were almost completely resistant. Since, in vivo, lymphocytes are exposed to even higher concentrations of the various serum components, it seems unlikely that ketoconazole would clinically act as an immunosuppressant. It remains to be seen whether the inhibitory effects of clotrimazole and miconazole on DNA synthesis can also be

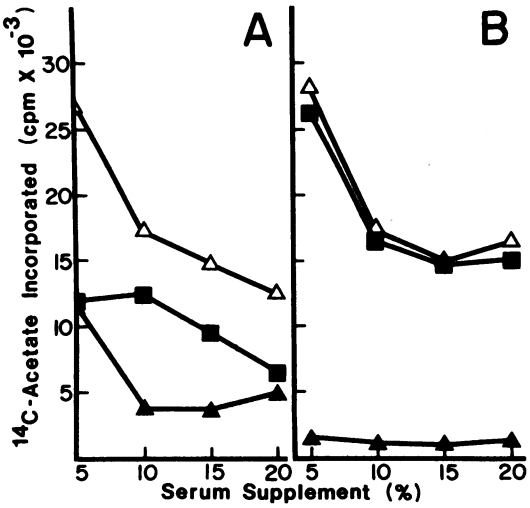

FIG. 8. Influence of AHS on the inhibition by ketoconazole of PHA-induced sterol synthesis. Human lymphocytes were cultured in 5, 10, 15, or 20% AHS in the absence (A) and presence (B) of 10 μg of ketoconazole per ml. Cells were labeled with [^{14}C]acetate, and labeled lipids were analyzed as described in the text. Symbols: △, total sterols; ■, cholesterol; ▲, lanosterol.

overcome by increasing the concentration of serum employed in vitro.

To determine whether B and T lymphocytes differed in their susceptibility to ketoconazole, we studied the effects of the drug on DNA synthesis and sterol synthesis in lymphocytes stimulated with mitogens specific for either B cells or T cells, as well as in purified populations of mouse B and T lymphocytes induced with the appropriate mitogen. In every case, the two cell types were found to be equally susceptible to ketoconazole. Previous studies have suggested that clotrimazole may preferentially inhibit specific T-cell populations (12), but our results do not indicate a similar cellular specificity for ketoconazole.

Apart from its effects on DNA synthesis, ketoconazole was also found to inhibit cholesterol metabolism in mitogen-induced lymphocytes. This finding was not totally unexpected because ketoconazole has been reported to inhibit lanosterol demethylation in cell-free liver homogenates (23, 24). However, it was surprising that cholesterol biosynthesis was 10-fold more susceptible to the drug than was DNA synthesis and that increasing the concentrations of AHS in the medium to a level which restored DNA synthesis (20%) did not overcome the block in cholesterol metabolism. These observations provide evidence for at least two distinct sites of action of ketoconazole within the lymphocyte, the cytochrome P-450-dependent lanosterol demethylase and another site leading to the block in DNA synthesis. Consistent with there being two or more sites for ketoconazole interaction, we found that the drug could be added as late as 55 to 60 h after the addition of mitogen and still completely suppress DNA replication. In both human (20) and mouse (6) lymphocytes, cholesterol synthesis increases transiently, with peak sterol production occurring 24 h after mitogen stimulation, followed by a rapid decline in cholesterol synthesis. Our studies showed that after PHA stimulation DNA synthesis peaked at 60 h in mouse lymphocytes and 72 h in human lymphocytes. By adding ketoconazole as late as 55 h after mitogen addition, the effects of the drug on cholesterol metabolism can be largely avoided, enabling us to determine the effects of ketoconazole on DNA synthesis directly. Under these conditions, the drug still inhibited DNA replication. Therefore, we propose that the inhibition of DNA synthesis by ketoconazole is not merely due to the ability of the drug to block cholesterol metabolism. In this regard, it is of interest that multiple target sites of ketoconazole activity have recently been proposed for the antifungal properties of the drug (22).

As mentioned earlier, the inability of 20% AHS to overcome the inhibition by ketoconazole of cholesterol synthesis demonstrates that AHS is neither inactivating nor complexing the drug in such a way as to effectively remove it from the cells. Rather, there appears to be a component(s) in AHS which can offset the effect of ketoconazole on DNA synthesis, but not its effect on cholesterol metabolism. The putative component is probably not cholesterol because the abilities of the various sera to restore DNA synthesis could not be correlated with their cholesterol content. Increasing the amount of HSA in the medium also did not rescue DNA replication, making it unlikely that this protein complexes with ketoconazole to prevent the entry of the drug into the cell. Lastly, the component is apparently nondialyzable since the lipoprotein-free serum and the mock-lipoprotein-free serum were capable of restoring DNA replication. Clearly, more work is necessary to elucidate the mechanism by which AHS can overcome the inhibition of DNA synthesis by ketoconazole.

Ketoconazole may prove valuable in defining the sterol requirements of mammalian cells. The results of several studies have suggested that lymphocytes require a period of endogenous cholesterol synthesis to progress from the G_1 phase to the S phase of the cell cycle (6, 20). However, this apparent requirement for cholesterol synthesis was demonstrated through the use of inhibitors which act primarily at the level of the 3-hydroxy-3-methylglutaryl coenzyme A reductase, an early step in sterol synthesis. Thus, it could not be established that the requirement was for cholesterol, per se, and not some other product of the isopentenoid pathway (e.g., dolichol). By contrast, ketoconazole acts at the level of lanosterol demethylation, a relatively late step in the cholesterol biosynthetic pathway. Our results showing that lymphocytes can synthesize DNA even when cholesterol synthesis is inhibited by greater than 90% indicate that cholesterol synthesis is not a prerequisite for entering the S phase of the cell cycle. This finding is in good agreement with the recent results of Cuthbert and Lipsky (8), who showed that endogenous cholesterol biosynthesis is needed for cell division, but not for DNA synthesis. Lanosterol has been shown to be inferior to cholesterol in supporting the growth of animal cells (5, 15) and in regulating membrane permeability and fluidity (16). Thus, it will be of interest to determine whether lymphocytes incubated in the presence of ketoconazole and 20% AHS are able to undergo mitosis and whether ketoconazole inhibits other immune processes involving lymphocytes.

In a recent study, Grosso et al. showed that ketoconazole can inhibit testosterone biosynthe-

sis in canine testes (13). The mechanism by which ketoconazole interferes with steroid hormone synthesis is not known, but our results suggest that the drug may be acting at the level of cholesterol biosynthesis. Due to the ease with which large numbers of relatively pure lymphocytes can be isolated from humans and mice, we believe that mammalian lymphocytes may provide an excellent model system for examining the effects of low concentrations of ketoconazole on animal cell sterol synthesis in general.

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