Amphotericin B and 5-Fluorocytosine: In Vitro Effects on Lymphocyte Function

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The effects of amphotericin B, 5-fluorocytosine, and the combination of both drugs on lymphocyte function in vitro were investigated. Amphotericin B, alone or in combination with 5-fluorocytosine, significantly suppressed both spontaneous lymphocyte transformation and the response of lymphocytes to stimulation with streptokinase-streptodornase. 5-Fluorocytosine had no effect on spontaneous or antigen-induced transformation. Lymphocyte responses to the mitogens phytohemagglutinin and concanavalin A were not changed by exposure to amphotericin B, 5-fluorocytosine, or the combination of both drugs. T-lymphocyte receptors for sheep erythrocytes and B-lymphocyte surface immunoglobulin and receptors for complement were not changed by treatment with amphotericin B or 5-fluorocytosine.

Amphotericin B (AmB) is a polyene antibiotic that binds to sterols in the fungal cell membrane, leading to leakage of cell contents and death of the cell (9). 5-Fluorocytosine (5-FC) is a fluorinated pyrimidine which is used almost exclusively in combination with AmB in infections caused by organisms such as *Candida*, *Torulopsis*, and *Cryptococcus*. Using in vitro assays for T-lymphocyte function and an in vivo murine model of T-cell function, several investigators have indicated that AmB may be immunosuppressive (19; F. L. Delmonico and R. Rubin, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 16th, Chicago, Ill., Abstr. no. 138, 1976; R. H. Rubin, R. B. Colvin, and M. E. Hammond, 17th ICAAC, New York, N.Y., Abstr. no. 273, 1977). However, immunostimulatory effects of AmB also have been reported in murine systems (4, 10, 11, 14, 15, 20). The effects of 5-FC and the combination of 5-FC and AmB on the immune system have not been studied.

The effects of these antifungal agents on the immune system may be of great significance in the treatment of patients with fungal diseases. Immunosuppression could be detrimental to successful treatment of fungal infections. On the other hand, immunostimulatory effects of antifungal antibiotics could prove to be a useful adjunct to host defenses against fungi. This study specifically evaluated the effect of AmB, 5-FC, and the combination of both drugs on B- and T-lymphocyte function in vitro.

MATERIALS AND METHODS

Antifungal drugs. AmB was obtained from E. R. Squibb & Sons, Inc., Princeton, N.J. To each vial containing 50 mg of sterile lyophilized AmB was added sterile distilled water so that the final concentration of AmB was 5,000 µg/ml. This stock solution was stored in aliquots at -70°C until used. Further dilutions were made with phosphate-buffered saline (PBS), pH 7.2.

5-FC powder (kindly supplied by Hoffman-La Roche, Inc., Nutley, N.J.) was diluted in PBS to a concentration of 5,000 µg/ml, filtered through a 0.45-µm membrane filter (Millipore Corp., Bedford, Mass.), and stored in aliquots at -70°C until used. Further dilutions were made with PBS.

T-cell rosette formation. Ficoll-Isopaque-separated mononuclear cells (5) from six healthy donors were washed three times in Hanks balanced salt solution (HBSS) and incubated in HBSS for 2 h at 37°C with concentrations of 1 and 5 µg of AmB per ml and 10 and 100 µg of 5-FC per ml. Control cells were incubated with PBS. The cells were then washed three times with HBSS, and T-cell rosettes were prepared by the method of Jondal et al. (12). A 0.25-ml quantity of cell suspension (4×10^6 cells per ml), 0.25 ml of sheep erythrocytes (10^8 cells per ml), and 1 drop of latex particles were mixed together. The mixture was incubated for 10 min at 37°C, spun at $200 \times g$ for 5 min, and placed at 4°C overnight. Those lymphocytes with three or more sheep erythrocytes attached were scored as T rosettes.

B-cell complement receptors and surface immunoglobulin. Washed Ficoll-Isopaque-separated mononuclear cells from six healthy donors were incubated in HBSS for 2 h at 37°C with 1 and 5 µg of AmB

per ml and 10 and 100 μg of 5-FC per ml. Control cells were incubated with PBS. Zymosan (Sigma Chemical Co., St. Louis, Mo.) was boiled in 10 volumes of 0.9% NaCl, centrifuged at $5,000 \times g$, washed three times with HBSS, and suspended at a concentration of 1 mg/ml. This suspension was mixed with fresh human serum for 30 min at 37°C (allowing fixation of complement), washed three times with HBSS, diluted to a final concentration of 0.5 mg/ml, and frozen at -70°C in 0.4-ml aliquots (16).

After incubation with the drugs, the lymphocytes were washed with HBSS, and 0.2 ml of a suspension of 4×10^6 cells per ml was mixed with 0.4 ml of complement-coated zymosan particles. The suspension was centrifuged at $200 \times g$ for 5 min and counted immediately. Lymphocytes with three or more zymosan particles attached were considered to have complement receptors.

For determination of surface immunoglobulin, 0.1 ml of cell suspension (2×10^7 cells per ml) was combined with 0.1 ml of fluorescein-conjugated goat anti-human immunoglobulin M (Meloy Laboratories, Springfield, Va.) following the 2-h incubation with drugs. This mixture was incubated at 4°C for 30 min, the cells were washed three times with cold HBSS, and the percentage of fluorescent cells was determined with a Zeiss standard microscope fitted with fluorescent accessories.

Lymphocyte transformation. Ficoll-Isopaque-separated mononuclear cells from 12 healthy donors were washed twice with Eagle minimum essential medium (Grand Island Biological Co., Grand Island, N.Y.) and suspended at a concentration of 5×10^5 cells per ml in Eagle minimal essential medium with 20% fetal calf serum (Grand Island Biological Co.), 1 mM L-glutamine, and 10 μg of streptomycin per ml added. Each glass culture tube (12 by 75 mm) received 1 ml of cell suspension. Quantities of 10 μl of varying concentrations of the following were added to the appropriate cultures: phytohemagglutinin-M (PHA; Difco Laboratories, Detroit, Mich.), undiluted to 1:30 dilution; concanavalin A (ConA; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), 2 to 20 μg /ml; and streptokinase-streptodornase (SK-SD; American Cyanamid Co., Lederle Laboratories Div., Pearl River, N.Y.), 4 to 40 U of SK. AmB was added to the appropriate cultures in concentrations varying from 0.2 to 10 μg /ml. 5-FC was added to other cultures in concentrations ranging from 10 to 200 μg /ml. Still other experiments had combinations of both drugs added to the cultures. Control cultures were done with

cells alone, cells with drug alone, and cells with mitogen or antigen alone. All cultures were done in triplicate.

After the appropriate incubation period (3 days for mitogens, 6 days for SK-SD), the cultures were pulse-labeled for 2 h with 1 μCi of [*methyl*- ^3H]thymidine (specific activity, 6 Ci/mmol). The amount of radioactivity incorporated into the acid-precipitable fraction was assayed in a Beckman liquid scintillation counter (13). Results were expressed as mean disintegrations per minute (DPM) or as the stimulation index, which is the ratio of DPM in stimulated cultures to DPM in control cultures. For each volunteer the dose of antigen or mitogen giving the maximum response was used for all calculations.

Statistical analysis. The data were evaluated with the Student *t* test for paired samples.

RESULTS

The expression of lymphocyte surface receptors and surface immunoglobulin was not altered by treatment with AmB or 5-FC. The percentages of T and B lymphocytes were the same in control and drug-treated mononuclear cell preparations (Table 1). Trypan blue dye exclusion showed >95% viability of all preparations after the 2-h incubation period with drugs.

Spontaneous lymphocyte transformation was significantly depressed by concentrations of AmB varying from 0.2 to 5.0 μg /ml ($P < 0.005$) (Fig. 1). Further studies showed that concentrations as low as 0.05 μg of AmB per ml suppressed spontaneous transformation by 40%. The mitogenic response, expressed as DPM, to both PHA and ConA was unchanged by the addition of AmB to the cultures. However, the response to the specific antigen SK-SD was significantly diminished ($P < 0.005$) by the addition of AmB.

In contrast to AmB, 5-FC, in concentrations from 10 to 200 μg /ml, had no effect on spontaneous transformation, mitogenic response to PHA or ConA, or the response to the specific antigen SK-SD (Fig. 2).

The combination of both AmB and 5-FC appeared to act in a fashion similar to that of AmB alone (Fig. 3). Spontaneous transformation and

TABLE 1. Percentage of T and B lymphocytes after *in vitro* exposure to AmB and 5-FC

Drug	Concn ($\mu\text{g}/\text{ml}$)	T lymphocytes (%)	B lymphocytes (%)	
			Surface immunoglobulin	C3 receptors
None		78.8 $\pm$ 2.6	4.2 $\pm$ 1.5	9.0 $\pm$ 2.2
AmB	1	75.5 $\pm$ 3.8	4.9 $\pm$ 1.7	8.3 $\pm$ 0.8
AmB	5	77.0 $\pm$ 3.8	5.0 $\pm$ 1.4	8.2 $\pm$ 1.1
5-FC	10	76.5 $\pm$ 3.2	5.0 $\pm$ 1.6	6.5 $\pm$ 2.2
5-FC	100	74.3 $\pm$ 4.2	5.3 $\pm$ 1.4	5.8 $\pm$ 0.7

FIG. 1. Effect of varying concentrations of AmB on spontaneous lymphocyte transformation (○) and on the response of lymphocytes to PHA (▲), ConA (△), and SK-SD (●), expressed as mean DPM for each concentration of AmB. An asterisk (*) indicates $P < 0.005$ (Student's t test for paired samples).

the response to SK-SD were both depressed to the same extent by the combination as they were with AmB alone ($P < 0.005$). The response to PHA and ConA was not significantly changed after exposure to the combination of AmB and 5-FC.

If the data are expressed in terms of stimulation index, it appears that AmB alone or in combination with 5-FC enhances the mitogenic response. However, this increase is due wholly to a drop in background spontaneous transformation and does not reflect a true increase in absolute DPM.

DISCUSSION

AmB is currently the mainstay of antifungal chemotherapy. Patients with disseminated fungal infections are frequently immunosuppressed as a result of their underlying diseases and also in some instances as a result of their overwhelming fungal infection (6, 18). Any further suppression of the immune response by the antifungal agent could prove detrimental to the eventual cure of the infection. Because of the multiplicity of factors influencing the immune system in

patients with serious fungal infections, we sought to assess the effects of AmB, 5-FC, and the combination of both drugs in a well-defined *in vitro* system.

In vitro, AmB significantly diminished both spontaneous transformation and the response to the specific antigen SK-SD. These effects occurred at concentrations of drug achievable in humans (3). In contrast to the studies of Tärnvik and Ånsén (19), we were unable to document an effect on the mitogenic response to PHA and ConA, even at concentrations as high as 10 µg/ml. This differential inhibitory effect on specific antigenic response but not on mitogenic response is similar to that reported after vaccination with live viral vaccines (8, 17) and after treatment with corticosteroids (2, 7) and cytotoxic drugs (1). However, it is possible that this difference could be related to the fact that cultures stimulated with antigens were incubated longer (6 days) than those incubated with mitogens (3 days).

Using different assays for cell-mediated immunity, Rubin et al. have documented immunosuppression in both mice and guinea pigs treated with AmB (16th ICAAC, Abstr. no 138;

FIG. 2. Effect of varying concentrations of 5-FC on spontaneous lymphocyte transformation (○) and on the response of lymphocyte to PHA (▲), ConA (△), and SK-SD (●), expressed as mean DPM for each concentration of 5-FC.

FIG. 3. Effect of the combination of AmB and 5-FC on spontaneous lymphocyte transformation (○) and on the response of lymphocytes to PHA (▲), ConA (△), and SK-SD (●), expressed as mean DPM for each combination of AmB and 5-FC. An asterisk (*) indicates $P < 0.005$ (Student's *t* test for paired samples).

17th ICAAC, Abstr. no 273). Others have shown AmB to be immunostimulatory to murine lymphocyte function both *in vitro* and *in vivo* (4, 10, 11). All studies showing AmB to have immunostimulatory effects on lymphocytes have used murine cells; immunostimulation has not been documented using human cells. Perhaps this difference in test systems explains the differing results found between our study and those of others (4, 10, 11).

5-FC had no effect on *in vitro* lymphocyte responses, and the combination of AmB and 5-FC showed no more suppression of lymphocyte transformation than that found with AmB alone.

Thus, in a well-defined and wholly *in vitro* system, AmB, but not 5-FC, diminished both spontaneous and antigen-induced lymphocyte transformation. In this system, AmB selectively

inhibited transformation to antigen and was not totally immunosuppressive. Whether this *in vitro* phenomenon has relevance to patients with serious fungal infections requiring AmB treatment is conjectural. Further *in vivo* studies in animal models of chronic antifungal therapy should be pursued to assess further the immunosuppressive potential of AmB.

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