Influence of Six Antifungal Agents on the Chemiluminescence Response of Mouse Spleen Cells

GEORGE K. ABRUZZO,[1]* DAVID M. GILTINAN,[2] THOMAS P. CAPIZZI,[2] AND ROBERT A. FROMTLING[1]

Department of Basic Microbiology[1] and Department of Biometrics Research,[2] Merck Institute for Therapeutic Research, Rahway, New Jersey 07065

The luminol-enhanced chemiluminescence (CL) assay is a measure of the early events of phagocytosis, leukocyte activation, and immune cell interactions. Reduction in the CL response of immune cells may be indicative of an inhibition of the immune response. This study was undertaken to examine the effects of antifungal agents tested at concentrations above and below therapeutically achievable levels on the CL response of mouse spleen cells. The effects of six antifungal agents—amphotericin B, ketoconazole, miconazole, 5-fluorocytosine, Bay-n-7133, and ICI 153,066—were studied. Changes in the CL response were assessed in terms of peak intensity and area under the intensity-time curve compared with appropriate diluent controls for each drug. Only amphotericin B and ketoconazole caused statistically significant lowering of the peak intensity at levels that are therapeutically attainable (mean peak plasma levels of 2 to 4 and 3.5 to 16 μg/ml, respectively). Although amphotericin B and ketoconazole caused reduction in the CL response, removal of the unbound drug in the preparation by centrifugation, washing, and suspension of cells in fresh drug-free medium resulted in a return of CL activity equivalent to the activity observed in cells not exposed to these agents. These results suggest that amphotericin B and ketoconazole at therapeutic concentrations may cause a reduction in immune cell antimicrobial activity; the clinical significance of these observations remains to be determined.

Normal function of phagocytic cells is an essential factor of proper host defense against invading microorganisms and is vital for successful defense against fungal agents of disease, since many of the drugs used in antifungal therapy have been shown to be fungistatic and not fungicidal (9). One of the early events of phagocytosis is the ability of immunocompetent cells to respond to appropriate stimuli by activation of the "respiratory burst," which is a coordinated series of metabolic reactions that leads to the generation of activated oxygen compounds that participate in the destruction of invading microorganisms (2, 3). An appropriate stimulus may be a fungal or bacterial cell, a particulate agent such as zymosan, or soluble agents such as the plant lectins phytohemagglutinin and concanavalin A. A sensitive way to measure the respiratory burst is based on the finding that during the generation of these activated oxygen compounds, a photon of light also is generated (2). This emitted light can be detected as chemical luminescence or chemiluminescence (CL), which is enhanced by the presence of luminol (1). The generation of CL has been shown to depend upon superoxide, hydrogen peroxide, singlet oxygen, hyroxyl radicals, and myeloperoxidase-catalyzed reactions (1, 23). Although the luminol-enhanced CL assay is an in vitro measure of the early events of phagocytosis, leukocyte activation, and immune cell interactions (1, 23), a reduction in the measured CL response of immune cells may be indicative of an inhibition of the immune response.

A number of reports have shown that several antibacterial agents at therapeutic concentrations can inhibit immune cell functions, including chemotaxis, uptake and killing of invading organisms, and certain metabolic functions as measured by the CL response (15, 21, 26). However, other reports have shown conflicting results with many of these same antibacterial agents (15, 16, 20). Notable among these anti-

bacterial agents are tetracycline, trimethoprim, sulfamethoxazole, cephalothin, and gentamicin.

A few antifungal agents, including amphotericin B and ketoconazole, have been examined for their effect on immune cell function. Amphotericin B has been shown to inhibit chemotaxis (4, 13, 18), phagocytic capacity and killing (5, 13), and CL (4, 13). One report has shown that ketoconazole and miconazole cause a significant reduction in the chemotaxis of polymorphonuclear neutrophils (7). Conflicting data also have been reported for these antifungal agents (21, 22).

The conflicting data reported for these antibacterial and antifungal agents are not surprising when the diversity of techniques and immune cell types which have been used are examined. The purpose of this study was to develop a simple and reliable method for standardizing this type of assay and to determine whether antifungal agents inhibit the respiratory burst of immune cells in vitro as measured by reduction in CL. We studied four commonly used antifungal agents (amphotericin B, ketoconazole, miconazole, and 5-fluorocytosine) and two experimental agents (Bay-n-7133 and ICI 153,066) for their effect on the generation of CL in mouse spleen cells in vitro.

MATERIALS AND METHODS

Antimicrobial agents. The antimicrobial agents tested included ketoconazole (Janssen R & D Inc., New Brunswick, N.J.), amphotericin B (Fungizone; E. R. Squibb & Sons, Princeton, N.J.), miconazole (Janssen R & D Inc.), 5-fluorocytosine (PCR Research Chemicals, Inc., Gainesville, Fla.), ICI 153,066 (Imperial Chemical Industries, Macclesfield, Cheshire, United Kingdom), Bay-n-7133 (Miles Pharmaceuticals, West Haven, Conn.), and rifampin (Calbiochem-Behring, La Jolla, Calif.). Ketoconazole, miconazole, ICI 153,066, Bay-n-7133, and rifampin were solubilized in dimethyl sulfoxide (DMSO) and brought to final

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* Corresponding author.

concentration with Dulbecco modified Eagle medium without phenol red (DMEM−PR; GIBCO Laboratories, Life Technologies, Inc., Chagrin, Ohio) supplemented with 10% fetal bovine serum (FBS; GIBCO). The DMSO concentration was such that a final concentration of 0.5% was obtained in the assay tubes. The agent 5-fluorocytosine was solubilized in DMEM−PR plus 10% FBS. Amphotericin B was a commercial preparation supplied as a sterile, lyophilized, desoxycholate-stabilized, phosphate-buffered powder. It was solubilized with sterile water according to the instructions of the manufacturer and then diluted in DMEM−PR plus 10% FBS. Drugs were tested, in part, at final concentrations that are considered clinically achievable in serum during therapy, and each drug was compared with its respective solubilizing agent.

Reagents. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St. Louis, Mo.) was solubilized at 1 mg/ml in phosphate-buffered saline (GIBCO) and 0.4% triethylamine (Aldrich Chemical Co., Inc., Milwaukee, Wis.). Zymosan A from *Saccharomyces cerevisiae* (Sigma) was suspended at 50 mg/ml in phosphate-buffered saline. Horseradish peroxidase (Sigma) was solubilized at 10 μg/ml in phosphate-buffered saline and prepared fresh on the day of use. Sodium desoxycholate (ICN Pharmaceuticals, Plainview, N.Y.) was suspended at 8.2 mg/ml in phosphate-buffered saline and then further diluted to 410 μg/ml in DMEM−PR plus 10% FBS.

Preparation of murine spleen cells. For each experiment, spleens from three 6- to 8-week-old BALB/cBY female mice (Jackson Laboratory, Bar Harbor, Maine) were aseptically removed, placed in 20 ml of chilled Hanks balanced salt solution (GIBCO), and then gently massaged between two sterile ground-glass microscope slides. The spleen cell suspension was passed through a 22-gauge sterile needle to further disrupt cell clumps, brought to a 40-ml volume by the addition of chilled Hanks balanced salt solution, washed three times in 40 ml of chilled Hanks balanced salt solution, and then suspended in 5 ml of chilled DMEM−PR plus 10% FBS. Cells were adjusted to a concentration of 10^7 cells per ml and incubated at 37°C in a 5% CO_2 atmosphere for a minimum of 30 min before use. Cell viability after preincubation with antifungal agents and their respective controls was determined by using a trypan blue exclusion method (26).

CL assay. For the CL assay, 470 μl of spleen cell suspension, adjusted to a concentration of 10^7 cells per ml, was aliquoted into Lumacuvettes (3M Medical Products Div., St. Paul, Minn.) and maintained at 37°C in a 5% CO_2 atmosphere until used. Next, 10 μl of concentrations with known titers of the test drugs or the diluent control was added to each tube, and the mixture was incubated for 30 min. After this incubation, 10 μl of luminol (1 mg/ml) was added, and the cells were incubated for an additional 10 min at 37°C. Background CL was monitored for 2 to 3 min, and then CL was induced by the addition of 10 μl of zymosan (50 mg/ml). CL response (intensity of emitted light in counts per minute) was measured with a six-channel Biolumat 9505 (Berthold, Wildbad, Federal Republic of Germany) interfaced with an Apple IIe computer and Epson RX-80 printer for data analysis and printing. The CL response was measured for each of the six treated groups every 20 s for 30 min. Each assay was performed a minimum of six times.

Wash protocol. Spleen cells were prepared as described previously and then preincubated with concentrations of determined titers of the test compound or diluent for 30 min at 37°C in a 5% CO_2 atmosphere. Cells then were collected by centrifugation at 1,000 × g for 10 min with a Centra-7R

centrifuge (International Equipment Co., Div. Damon Corp., Needham Heights, Mass.) and no. 216 rotor (International Equipment Co.), and washed twice in an equal volume of fresh medium without any drug. The washed cells were suspended in an equal volume of DMEM−PR plus 10% FBS, and the CL response was measured as described previously.

Cell-free quench protocol. Added to cuvettes was 470 μl of DMEM−PR plus 10% FBS, 10 μl of luminol (1.0 mg/ml), and 10 μl of horseradish peroxidase (10 μg/ml). The mixture was incubated at 37°C for 5 to 10 min; the Biolumat was then started, and the reaction was allowed to proceed for 1 min to obtain background counts. Then 10 μl of a 6 × 10^{-3} M solution of hydrogen peroxide (Fisher Scientific Co., Pittsburgh, Pa.) was added, and the reaction was allowed to proceed for approximately 4 min. The Biolumat was stopped, and 10 μl of test compounds was added. The Biolumat was restarted, and the reaction was monitored for a total of 10 min. The test compounds were solubilized as described previously and compared with their respective solubilizing agents for changes in the CL response after addition of the test compound. Data were analyzed from the time drugs were added as described below.

Spleen cell quench protocol. To cuvettes containing spleen cells at a concentration of 10^7 cells per ml, 10 μl of luminol (1 mg/ml) was added, and the mixture was incubated at 37°C for 10 min. The Biolumat was started, and the reaction was allowed to proceed for 2 to 3 min to obtain background counts; 10 μl of zymosan (50 mg/ml) was then added, and the reaction was allowed to continue for 12 to 18 min. The Biolumat was stopped, and 10 μl of test compound was added. The Biolumat was restarted, and the reaction was monitored for a total of 25 min. Data were analyzed from the time the drugs were added as described below.

Analysis of data. The experiments were designed so that the allocation of treatments was balanced over factors found to have a systematic effect on the CL response, thereby allowing an unbiased evaluation of the effect of treatment. A 6 by 6 Latin square design was used for this purpose (6). Three parameters were used when analyzing the CL response: peak intensity of the emitted light, time to peak, and the area under the intensity-time curve. The data indicated that peak intensity alone provides an adequate characterization of the CL response (11). Since percent change in this response after treatment was of interest, it was analyzed on the natural log (log_e) scale. An overall assessment of the difference in mean response at the various levels of the test compound was performed by means of an F test for treatment differences in an analysis of variance. A more detailed evaluation of the effect of treatment was carried out with Dunnett's test (8), comparing each concentration of the test compound with the control, and with the trend analysis of Tukey et al. (24), which tests for progressiveness of response with increasing concentration of the test compound.

RESULTS

Effects of antifungal agents on the CL response of spleen cells from BALB/cBY mice. Preliminary kinetics studies determined that CL after stimulation with zymosan was dependent on spleen cell concentration. A spleen cell concentration of 10^7 cells per ml was selected for these studies, since small changes in CL kinetics could best be determined at this cell concentration.

Since the azole compounds and rifampin were solubilized in DMSO, it was first necessary to determine the effects of DMSO on spleen cell CL after stimulation with zymosan.

After preincubation of cells for 30 min with DMSO concentrations of determined titers, the response was measured and statistically analyzed. Preincubation with 2% DMSO for 30 min significantly reduced the CL response of BALB/cBY spleen cells, while levels of 1% or less did not significantly reduce the CL response. A level of 0.5% DMSO was used based on these results and the solubility of all the drugs at this concentration. A concentration of desoxycholate (8.2 mg/ml) in phosphate buffer diluted to a final test concentration of 8.2 μg/ml in DMEM–PR plus FBS (equivalent to that in the highest assayed level of amphotericin B) was tested and found to have no significant effect on the CL response. None of the antifungal agents tested or their respective controls caused significant cell death as determined by trypan blue exclusion.

Amphotericin B was assayed at concentrations from 0.625 to 10 μg/ml by twofold dilutions. After preincubation at these levels of amphotericin B for 30 min, a statistically significant reduction in CL response of mouse spleen cells was observed at all but the lowest concentration ($P < 0.05$; Table 1). Peak plasma levels in humans for amphotericin B are reported to be from 2 to 4 μg/ml (17). Thus, at levels which are therapeutically achievable, amphotericin B significantly reduced the CL response of mouse spleen cells as determined in this in vitro system.

Ketoconazole was tested at concentrations of 0.08 to 20 μg/ml by twofold dilutions. At concentrations of 0.08 to 2.5 μg/ml, ketoconazole did not significantly affect spleen cell CL (Table 2). Although the 2.5-μg/ml concentration did not cause a statistically significant reduction in peak CL, P for this comparison with the control was 0.0549. We retested ketoconazole at concentrations of 1.0 to 5.0 μg/ml by 1-μg/ml increments. The results show that ketoconazole at levels of 3.0 to 20 μg/ml gave a statistically significant reduction in the peak CL response ($P < 0.05$; Table 2). Ketoconazole has been reported to attain plasma levels in humans of 3.5 μg/ml (17) and higher (8 to 16 μg/ml) in patients receiving the high doses administered for certain clinical forms of coccidioidomycosis (A. M. Sugar, D. A. Stevens, S. Alsip, W. E. Dismukes, J. N. Galgiani, J. R. Graybill, and P. C. Craven, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1100, 1984).

The agent 5-fluorocytosine had no effect on the CL response of mouse spleen cells at concentrations up to 20 μg/ml (Table 1). Of the other three drugs tested, miconazole

TABLE 2. Effects of ketoconazole on the CL response of spleen cells from BALB/cBY mice

Ketoconazole concn (μg/ml)	Geometric mean peak intensity (cpm, 10^3)
0	6,317
0.08	6,438
0.16	6,176
0.31	6,256
0.625	6,166
1.25	6,253
0	2,978
1.25	2,705
2.5	2,597
5.0	2,345[a]
10.0	2,008[a]
20.0	1,265[a]
0	1,516
1	1,388
2	1,386
3	1,210[a]
4	1,122[a]
5	1,128[a]

[a] Statistically significant reduction in peak CL compared with that of diluent control (no drug), as determined by Dunnett's test ($P < 0.05$) and the trend test of Tukey et al.

and ICI 153,066 reduced the peak CL response of mouse spleen cells at 20 μg/ml (Table 1). Bay-n-7133 caused no significant reduction in peak CL at concentrations up to and including 20 μg/ml when the data were analyzed with Dunnett's test. However, at the 10- to 20-μg/ml concentrations, mean responses were lower, and a trend analysis indicated a significant negative response trend with increasing concentration of the drug (Table 1). The indication is that higher concentrations have a lowering effect on the CL response, even though the reduction is not found to be significant by the (less sensitive) Dunnett's test. Although mean peak plasma levels for Bay-n-7133 in mice are between 14 and 16 μg/ml (19), the peak plasma level in humans is reported to be only 2.76 μg/ml (25), a value below the concentration determined to cause a significant reduction in CL activity in our assay. In all other instances, data analyzed by both the trend test and Dunnett's test agreed. Peak plasma levels in humans for miconazole are reported to be above 1 μg/ml (17), and peak plasma levels for ICI 153,066 have not been reported.

Effect of removal of antifungal agents by centrifugation and washing. Spleen cells that were preincubated with antifungal agents for 30 min were collected by centrifugation, washed twice, suspended in fresh medium without drugs, and then assayed. These CL data were compared with the CL data from cells treated the same way but without exposure to drugs. Reduction in the CL response of washed cells was compared with reduction observed in drug-exposed, nonwashed cells. Amphotericin B was tested at 10 μg/ml. Ketoconazole was tested at 20 and 10 μg/ml. Miconazole, Bay-n-7133, and ICI 153,066 were assayed at 20 μg/ml. The agent 5-fluorocytosine was not tested, since it caused no inhibition at concentrations up to 20 μg/ml. The data show that removal of unbound ketoconazole with this method resulted in a return to the level of CL activity observed in cells not exposed to the drug (Fig. 1). Data from washing experiments with the other antifungal agents show similar results.

Cell-free quench assay. These experiments were performed

TABLE 1. Effects of antifungal agents on the CL response of BALB/cBY spleen cells

Concn (μg/ml)	Geometric mean peak intensity (cpm, 10^3)				
	Amphotericin B	5-Fluorocytosine	Miconazole	Bay-n-7133	ICI 153,066
0	9,672	10,961	7,771	3,262	8,045
0.625	9,458	NT[a]	NT	NT	NT
1.25	8,982[b]	11,069	7,447	3,297	7,939
2.5	8,400[b]	11,222	8,004	3,340	8,138
5.0	7,607[b]	11,140	7,962	3,296	7,634
10.0	6,594[b]	10,870	8,161	3,074[c]	7,659
20.0	NT	11,260	5,844[b]	3,008[c]	6,363[b]

[a] NT, Not tested.
[b] Statistically significant reduction in peak CL compared with that of diluent control (no drug) as determined by Dunnett's test ($P < 0.05$) and the trend test.
[c] Statistically significant reduction in peak CL compared with that of diluent (no drug) as determined by the trend analysis of Tukey et al. ($P < 0.05$) alone.
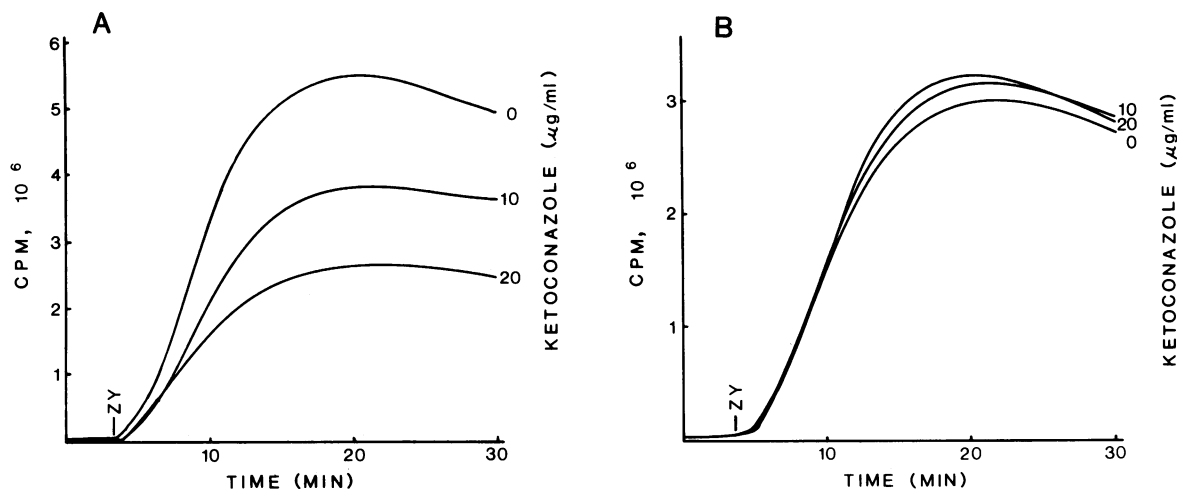
FIG. 1. Effect of removal of ketoconazole by centrifugation and washing. (A) Effect of preincubation of BALB/cBY spleen cells with 10 and 20 μg of ketoconazole per ml for 30 min on the CL response after stimulation with zymosan (ZY). (B) CL response after stimulation with zymosan of BALB/cBY spleen cells that had been preincubated with 10 and 20 μg of ketoconazole per ml for 30 min after the drug was removed by centrifugation, washing, and suspension in fresh medium without drug. Graphs display representative data.

to exclude absorption of emitted light (quenching) by the antifungal agents as the cause of reduction in the CL response. Initially, a cell-free assay with luminol, horseradish peroxidase, and hydrogen peroxide was used to test for quenching, because it was felt that a simple system without the cells might give clearer results. Rifampin was used as the positive control, since reports have shown that it has a quenching effect on emitted CL (21). Preliminary experiments were performed to determine the effects of rifampin in this cell-free system. Rifampin was tested at concentrations ranging from 20 to 1.25 μg/ml by twofold dilutions. Addition of rifampin during the height of the CL response caused an immediate drop in emitted CL, as compared with that of the 0.5% DMSO control for all rifampin levels tested. A level of 20 μg/ml was used as a positive control in subsequent tests.

Amphotericin B was tested at 10 μg/ml, and all of the other antifungal agents were assayed at 20 μg/ml and compared with their respective solubilizing agents. Only rifampin caused a statistically significant reduction in CL. None of the antifungal agents at the concentrations tested reduced CL due to absorption of emitted light (quenching) as determined in this assay (Table 3).

Spleen cell quench assay. A quench assay with BALB/cBY spleen cells was subsequently used to determine if similar results would be seen in the presence of cells. Rifampin at 20 μg/ml was used as the positive control. Amphotericin B was tested at 10 μg/ml, and all other drugs were tested at 20 μg/ml. Results were similar to those obtained with the cell-free quench assay. Only rifampin caused a statistically significant reduction in the CL response. None of the antifungal agents had a quenching effect at the concentrations tested.

DISCUSSION

CL is the light emitted by stimulated phagocytic cells during the generation of active oxygen compounds in the respiratory burst (2). CL is closely associated with oxidative metabolism and phagocytosis and results in the generation of active antimicrobial products that serve as the first line of defense of immune cells against invading microorganisms (2, 3). CL may serve as an indicator of the capability of immune

cells to respond to antigenic stimulation (2, 3, 23). Thus, reduction in the CL response of phagocytic cells may be indicative of inhibition of the immune response.

In this study, a luminol-enhanced CL assay was used to evaluate antifungal agents at concentrations above and below therapeutically attainable levels for their effect on the CL response of mouse spleen cells. Of the six antifungal agents tested, only amphotericin B and ketoconazole caused statistically significant lowering of the peak CL response at levels that are therapeutically attainable. Miconazole, ICI 153,066, Bay-n-7133, and 5-fluorocytosine did not cause statistically significant reduction in CL at therapeutic concentrations (Table 4). For all of the antifungal agents tested, reduction in the peak CL was not a result of absorption of emitted light (quenching) or cell death due to these agents. Although many of these antifungal drugs may bind to fungal sterol membranes, in all cases the removal of an unbound drug in the preparation by centrifugation resulted in a return

TABLE 3. Cell-free quenching of the CL response

Drug[a] in control group	Geometric mean peak intensity (cpm, 10[3])	% of group control during CL[b] (peak response)
0.5% DMSO	204,054	100
Ketoconazole	179,375	88
Rifampin	12,236[c]	6
Bay-n-7133	218,750	107
Miconazole	194,492	95
ICI 153,066	204,046	100
DMEM − PR + 10% FBS	125,373	100
Amphotericin B	120,017	96
5-Fluorocytosine	137,695	110
0.5% DMSO	130,561	100
Rifampin	4,734[c]	4

[a] All drugs at concentration of 20 μg/ml except amphotericin B at 10 μg/ml.

[b] Ratio of counts per minute with the drug to counts per minute with the diluent control (0.5% DMSO or DMEM − PR plus 10% FBS).

[c] Statistically significant (P < 0.05) reduction in peak CL as determined by Dunnett's test.

of CL activity equivalent to the activity observed in cells not exposed to these agents.

Data analysis from preliminary trials made obvious systematic differences among the measurement channels of the Biolumat. Thus, it was necessary to balance the allocation of treatments across channels of the machine as much as possible to avoid biasing the results. A 6 by 6 Latin square design (6) in which each treatment appeared exactly once in each of the measurement channels of the Biolumat was used in all experimental assays to eliminate this bias. We found the "treatment-by-day" interaction to be negligible, which indicated that the effects of the antifungal agents observed were consistent over time and among cell batches used in the assay. However, we did see variation in the intensity of background CL response measured in counts per minute from different cell batches. It has been reported that a considerable range in the CL response of human polymorphonuclear leukocytes may be encountered with cells from different donors (14). Also, we encountered systematic variation in counts per minute from assay to assay. This may be explained, in part, by the age of the cells, since the average background response for assays later in the day tended to be lower than that for assays performed earlier in the day, when the cells had been recently removed from the mice. These systematic variations do not enter into an assessment of the treatment effect, however, since each treatment appeared once in each experimental assay because of the 6 by 6 Latin square design. This balance allows an unbiased evaluation of the treatment effect and increases the precision of the evaluation, since the systematic channel-to-channel and assay-to-assay variation may be subtracted out of the residual variation during statistical testing. If reduction in the CL response is to be used as an indicator of possible inhibition of the immune response, then it is absolutely necessary to have a standardized method of statistically sound experimental design to eliminate potential bias inherent in the assay. We are proposing that the statistical methods described by Giltinan et al. (11), which were used in the present study, be used in the design and analysis of experiments of this type.

The need for a standardized procedure to evaluate the effects of antifungal agents on the CL response of immune cells becomes evident from a review of conflicting reports. An early report on the effects of amphotericin B on the CL response of human neutrophil chemotaxis and CL was presented by Bjorksten et al. in 1976 (4). They reported inhibition of the CL response after pretreatment of cells with

5 to 20 μg of amphotericin B per ml and stimulation with opsonized zymosan. They noted that these concentrations were higher than those therapeutically achievable in humans and that further studies would be required to determine clinical significance. A later study by Supapidhayakul et al. (22) reported that a commercially available colloidal preparation of amphotericin B actually stimulated the CL response of both human and canine polymorphonuclear cells but that purified amphotericin B, lacking the colloidal consistency, did not stimulate the CL response. It appears as though the colloidal particles in the commercial preparation were, in fact, the stimulating agent and not the amphotericin B. Siegel and Remington (21) reported no effect of amphotericin B at a concentration of 6.25 μg/ml on the CL response of polymorphonuclear cells stimulated with opsonized Candida albicans cells. Amphotericin B methyl ester has been reported to inhibit human neutrophil CL (13) to a greater degree than amphotericin B does. Ketoconazole at concentrations from 1 to 20 μg/ml has been reported to have no effect on the generation of CL by zymosan-stimulated polymorphonuclear cells (13). Our data show inhibition of the CL response of mouse spleen cells with ketoconazole at concentrations of 3.0 to 20 μg/ml. Similar conflicting data have been reported for certain antibacterial agents evaluated for their effects on immune cells, including the CL response (15, 16, 20, 21, 26). The conflicting reports on the effects of antimicrobial agents on CL may be the result of different methodologies, stimulating agents, and the use of different cell species and types.

Since many of the drugs used to treat fungal infections have been shown to be fungistatic and not fungicidal (9), elimination of the invading organism and complete recovery from the fungal disease may depend in large part on the immune system of the host. Patients with reduced immunological capability due to disease or certain forms of chemotherapy are more susceptible to opportunistic fungal infection (10, 12). When administered to neutropenic patients, drugs that inhibit the CL response may further reduce the already-impaired immune response of the host. The results presented in this study suggest that amphotericin B and ketoconazole at therapeutic concentrations may cause a reduction in immune cell antimicrobial activity in vivo. Ideally, an antifungal antibiotic should not act to reduce or suppress the immune response. Realistically, considering the limited number of effective antifungal therapeutic agents available, drugs such as amphotericin B and ketoconazole, despite their negative side effects, are very effective for the treatment of certain forms of fungal disease. Although our results show that both amphotericin B and ketoconazole cause a significant reduction in the CL response of mouse spleen cells at concentrations that are therapeutically achievable, it must be kept in mind that our assay is an in vitro system. Whether these in vitro results are significant in vivo remains to be determined.

TABLE 4. Statistical significance of effects of antifungal agents on the CL response of BALB/cBY spleen cells

Antifungal agent	Statistical significance at concn (μg/ml):							
	20	10	5	4	3	2.5	1.25	0.625
Amphotericin B	NT[a]	+[b]	+	NT	NT	+	+	−[c]
Ketoconazole	+	+	+	+	+	−	−	−
5-Fluorocytosine	−	−	−	NT	NT	−	−	NT
Miconazole	+	−	−	NT	NT	−	−	NT
Bay-n-7133	*[d]	*	−	NT	NT	−	−	NT
ICI 153,066	+	−	−	NT	NT	−	−	NT

[a] NT, Not tested.
[b] Statistically significant reduction in peak CL compared with that of diluent control (no drug) as determined by Dunnett's test (P < 0.05) and trend analysis of Tukey et al.
[c] Not significant by either test.
[d] Statistically significant reduction in peak CL compared with that of diluent control (no drug) as determined by the trend analysis of Tukey et al. (P < 0.05) alone.

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