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Caspofungin reduces the incidence of fungal contamination in cell culture

Luis R. Martinez · Patricia Ntiamoah · Arturo Casadevall · Joshua D. Nosanchuk

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Abstract Fungal contamination is a major problem in cell culture, and the antifungal compounds currently in use can affect cultured cells. Echinocandins are antifungal drugs that inhibit fungal cell wall synthesis by targeting an enzyme that has no counterpart in mammalian cells. We evaluated whether the echinocandin caspofungin affected the growth or morphology of six murine cell lines (a macrophage-like cell line (J774.16) and five hybridoma lines), or primary human endothelial cells. The antifungal did not influence cellular characteristics at concentrations less than 512 μg/ml, while effectively reducing the incidence of fungal contamination. Also, caspofungin did not affect the production of antibody by hybridoma cells, or alter the cytokine production of J774.16 cells, although modest increases in IL-4 and IFN-γ occurred upon LPS stimulation. Hence, echinocandins appear to be relatively non-toxic, and protect against fungal contamination in cell culture.

L. R. Martinez · P. Ntiamoah · A. Casadevall · J. D. Nosanchuk (✉)
Department of Medicine (Division of Infectious Diseases), Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
e-mail: nosanchu@aecom.yu.edu

L. R. Martinez · P. Ntiamoah · A. Casadevall · J. D. Nosanchuk
Department of Micriobiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA

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Introduction

Prevention of microbial contamination is a major concern for individuals and companies utilizing tissue culture techniques. Although strict sterile practice is the mainstay of handling cell lines, antibacterial agents such as penicillins and aminoglycosides are routinely used by many investigators and companies. In contrast, antifungal compounds are less commonly used. A recent report from a consortium of cell banks showed that 39% of specimen were contaminated over a 2 year period and fungi were identified in 8% of these [1]. Although both yeast and mold contamination occur, the most frequently isolated fungi are *Aspergillus* spp. [1, 2].

Amphotericin B and nystatin have historically been used for reducing fungal contamination in cell cultures [3]. Both of these polyene macrolide drugs target ergosterol in fungal cell membranes, which is sufficiently similar to cholesterol in structure such that damage can occur to mammalian cells [4]. Additionally, these compounds can stimulate the activity of diverse membrane enzymes and cellular metabolism [5]. Amphotericin B has also been shown to interact with Toll-like receptors and CD14 to induce signal transduction and release of inflammatory cytokines [6], and it is likely that nystatin can

function similarly. Hence, there are significant drawbacks of using either of these compounds in cell culture; particularly in immunological studies, where drug effects on cell function can be a confounding variable in experimental design and interpretation.

Caspofungin is an echinocandin drug that is used clinically for invasive fungal disease and functions by disrupting 1-3-β-D-glucan synthesis in the fungal cell wall by non-competitive inhibition of the enzyme 1-3-β-D-glucan synthase [7]. For instance, caspofungin has been effective against a very large collection of clinical isolates of *Aspergillus* spp., and are active in vitro against numerous other contaminating molds, including *Penicillium* and *Paecilomyces* spp. [8]. To our knowledge, echinocandins have no direct toxicity to cell lines at physiologic concentrations. We therefore sought to determine the safety and efficacy of the use of caspofungin as a proof of principle for their application in the culture of cell lines, and to compare it with amphotericin B.

Materials and methods

Cell lines and media

Six cell lines were studied. The macrophage-like cell line J774.16 was derived from a reticulum cell sarcoma, and has been extensively used to study pathogenesis of intracellular organisms [9]. The hybridoma cell lines examined were 6F12 (IgG2a), 7C7 (IgM), 9C7 (IgM), 3B9 (IgM), and 5B8 (IgM), which produce antibody to a cell surface antigen on *Histoplasma capsulatum* [10]. The monoclonal antibody producing cells were generated from the fusion of BALB/C splenocytes with SP2/0 or NSO cells [10]. All cells were grown in a standard medium {10% FBS: Dulbecco's modified Eagle's medium with L-glutamine (DMEM: GIBCO, Grand Island, NY), containing 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Woodland, CA0) 10% NCTC-109 without L-glutamine (GIBCO), and 1% nonessential amino acids (GIBCO)} with or without caspofungin, at 37°C with 10% CO_2 in 96-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ).

Endothelial cells and media

Primary human endothelial cells were obtained from umbilical cord veins perfused with type II collagenase (2 mg/ml; Worthington Biochemical, Freehold, NJ) to release the endothelial cells from the vessel wall. Then the cells were grown on 0.2% gelatin (Fisher Scientific)-coated tissue-culture plates in M199 medium supplemented with 20% newborn calf serum (NBCS), 5% heat-inactivated human serum (Gibco BRL), 1% penicillin–streptomycin, and 12 ng/ml EC growth factor (Sigma). Cultures were >99% factor VII positive, as demonstrated by immunofluorescence using an anti-factor VIII-related antigen antibody (IgG_1; 1:100; Dako), and nonreactive for an isotype-matched negative control (IgG_1 mouse myeloma protein 1:50; Organin Tecknica).

Comparison of susceptibility of cell lines to caspofungin and amphotericin B

To evaluate the effects of antifungal drugs on the appearance of J774.16 macrophage-like cells and the hybridomas, each cell line was suspended at 5×10^5 cells per ml in standard medium containing caspofungin (Merck, Whitehouse Station, NJ) (64, 128, 256, 512, or 1024 μg/ml) or amphotericin B (Gibco, Carlsbad, CA) (0.5, 1, 2, 4, or 8 μg/ml). The drug concentrations chosen for this study were above the MIC for several fungal species. Wells containing cells grown with medium alone were used as a control. For each cell line, 100 μl of the suspension was added into individual wells of polystyrene 96-well plates (Fisher), and were incubated at 37°C with 10% CO_2 for 24 h. After 24 h of incubation, growth rates and viability were determined by Trypan blue exclusion and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium-hydroxide (XTT) reduction assay. Briefly, 50 μl of XTT salt solution (1 mg/ml in PBS) and 4 μl of menadione solution (1 mM in acetone; Sigma) were added to each well. Microtiter plates were incubated at 37°C for 5 h. Fungal mitochondrial dehydrogenase activity reduces XTT tetrazolium salt to XTT formazan, resulting in colorimetric change that correlates with cell viability [11]. The colorimetric change was measured using a microtiter reader (Labsystems Multiskan MS; Labsystems, Finland) at 492 nm. Furthermore, microscopic examinations of the cell's morphology were performed by light microscopy with an Axiovert 200 M inverted microscope (Carl Zeiss MicroImaging).

Efficacy of caspofungin in reducing fungal contamination

To evaluate the efficacy of caspofungin in reducing fungal contamination, cell culture medium was placed into 96 well tissue culture plates (Becton Dickenson). Plates containing either medium alone, or medium with caspofungin (64 or 256 μg/ml) or amphotericin B (0.5 or 2 μg/ml) were incubated at 37°C with 10% CO_2. There were 6 plates for each condition. The plates were evaluated microscopically three times a week for evidence of contamination. Similarly, to simulate a breach in sterile technique, a second set of 6 plates for each condition was opened twice per week on the bench top for 1 min after microscopic survey, and incubated and evaluated as described above. Most common contaminating fungi have MIC_{50} of 0.03 μg/ml and 1 μg/ml for caspofungin and amphotericin B, respectively [12].

Effect of caspofungin in antibody production by hybridoma cells

The production of antibody in hybridoma cells was assessed in the presence and absence of caspofungin or amphotericin B. The cells were cultured for 10 days in 6-well tissue culture plates (Becton Dickinson) medium alone, or medium supplemented with 64 and 256 μg/ml of caspofungin or 0.5 and 2 μg/ml of amphotericin B. The supernatants were collected, and then the concentrations of antibody were determined by ELISA, as described elsewhere [10].

Cytokine/chemokine production of macrophages treated with caspofungin

A density of 5×10^5 J774 cells per well were cultured in 6-well plates in media alone, media with LPS, media alone or with LPS and 256 μg/ml of caspofungin, and media alone or with LPS and 2 μg/ml of amphotericin B. LPS was utilized to stimulate J774 cells and compare cytokine production of stimulated cells relative to unstimulated cells, since many researchers utilize LPS stimulated cells. Control wells consisted of medium without cells since the medium may contain cytokine traces from FCS used in medium preparation. Then, these results were subtracted form medium containing J774 cells and compared with experimental conditions. After 24 h, the medium was collected and cytokine/chemokine analyses were performed with the RayBio mouse cytokine antibody array I kit (RayBiotech, Inc., Norcross, GA) according to the direction of the manufacturer. The immunoregulatory molecules assayed were GCSF, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17, IFN-γ, MCP-1, MCP-5, RANTES, SCF, TNF-α, Thrombopoetin, and VEGF. The membranes were developed radiographically and ImageJ (http://rsb.info.nih.gov/ij/) was used to analyze the dot blot results. The relative protein levels were obtained by subtracting the background staining and normalizing to the positive controls on the same membrane.

Since the antibody array was qualitative, quantitative analysis of TNF-α and IFN-γ in supernatants was performed using cytokine specific ELISAs (Becton Dickinson Biosciences Pharmingen, San Diego, California, USA; and R&D Systems Inc., Minneapolis, Minnesota, USA). The detection limits of the cytokine assays were 15.6 pg/ml for TNF-α, and 31.3 pg/ml for IFN-γ, as stated by the manufacturer.

Statistical analysis

All data were subjected to statistical analysis using Origin 7.0 (Origin Lab Corp., Northampton, MA). *P* values were calculated by Student's *t* test or analysis of variance depending on the data. *P* values of <0.05 were considered significant.

Results

Caspofungin did not alter growth or morphology of cells at physiological concentrations

We evaluated whether caspofungin affected the growth or morphology of a macrophage-like cell line and two hybridoma cell lines. There was no difference in appearance between J774.16 cells grown without (Fig. 1A) or with 64 μg/ml caspofungin (Fig. 1B), or 0.5 μg/ml of amphotericin B (Fig. 1D). In contrast, J774.16 cells displayed morphological alterations and growth inhibition at drug concentrations ≥512 μg/ml of caspofungin (Fig. 1C) or ≥2 μg/ml of amphotericin B (Fig. 1E).

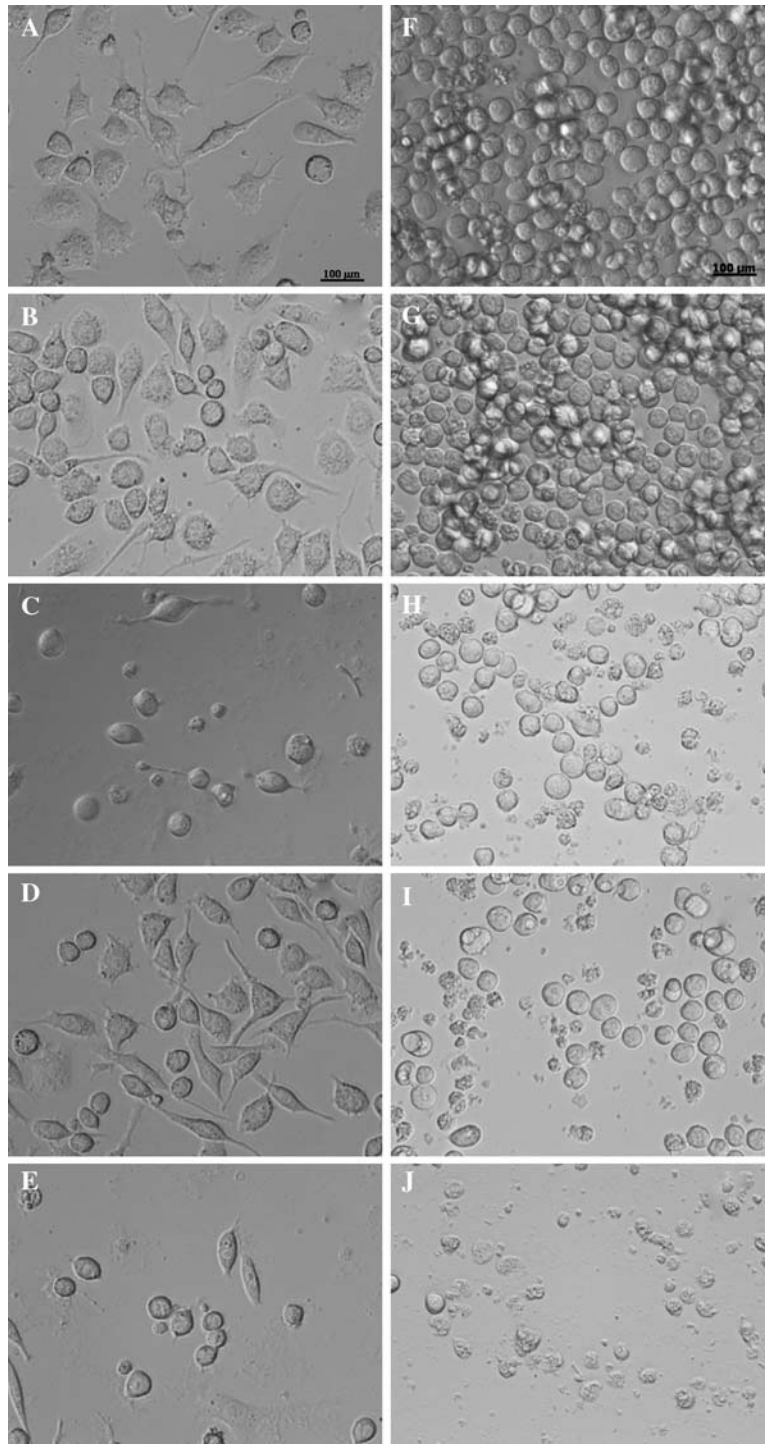

Fig. 1 Microscopic appearance of macrophage-like J774.16 and hybridoma 6F12 cells. J774.16 cells grown with cell culture medium alone (**A**), or supplemented with 64 (**B**) and 512 (**C**) µg/ml of caspofungin, or with 0.5 (**D**) and 2 (**E**) µg/ml of amphotericin B. Hybridoma 6F12 cells grown with cell culture medium alone (**F**), or in the presence of 64 (**G**) or 256 (**H**) µg/ml of caspofungin, or with 0.5 (**I**) and 2 (J) µg/ml of amphotericin B. Similar results occurred with the other hybridoma lines. Experiments were performed twice to thrice with reproducible results. Original magnification 400×

For the hybridoma cells, morphological alterations were noted for caspofungin concentrations ≥512 µg/ml (Fig. 1H) and amphotericin B concentrations ≥0.5 µg/ml (Fig. 1I). Cellular disruption was significantly observed at amphotericin concentrations of 2 µg/ml (Fig. 1J). Moreover, both drugs have similar effect on human endothelial cells (data not shown).

Growth rates and viability, as determined by Trypan blue exclusion and XTT reduction assay, were not affected until the concentration of caspofungin ≥512 μg/ml or amphotericin B ≥0.5 μg/ml for each cell line examined (data not shown). The studies of cell morphology, growth rates, and viability were repeated at least twice with similar results occurring each time.

Caspofungin reduced the incidence of fungal contamination

We explored whether caspofungin reduced the frequency of fungal contamination in plates aseptically handled within a laminar flow hood (Fig. 2A). In one of the repetitions of the experiment, the first contamination occurred at day 42 in the medium with 0.5 μg/ml of amphotericin B followed by the control group at day 49. In contrast, plates containing 64 or 256 μg/ml of caspofungin became contaminated by days 84 and 119, respectively. Microtiter plates with fungal growth were treated with cupric sulfate, and plates were discarded after half of the wells were contaminated. At day 115, all control plates and all but one of the 0.5 μg/ml of amphotericin B plates were contaminated. However, only one plate from each of those treated with 2 μg/ml of amphotericin B or caspofungin at 64 and 256 μg/ml had microbial growth. The efficacy of caspofungin over such a prolonged incubation time is remarkable, given that the drug can undergo spontaneous degradation to an open-ring peptide, and is also slowly metabolized by hydrolysis and N-acetylation. Similarly, amphotericin B half-life in tissue culture is 12 to 16 h, possibly due to drug degradation.

To simulate a breach in sterile technique, additional sets of 6 plates for each condition were opened on the bench top for 1 min after microscopic survey (Fig. 2B). The first contamination was observed at day 7 in the medium control group, and at days 23 and 27 in the plates containing amphotericin B concentrations of 0.5 and 2 μg/ml, respectively. In contrast, plates with concentrations of 64 or 256 μg/ml caspofungin were not contaminated until days 35 and 40, respectively. In general, minimal fungal growth was subsequently observed in the caspofungin plates, whereas the majority of wells in the control plates were infected by day 28. Likewise, 4 out of 6

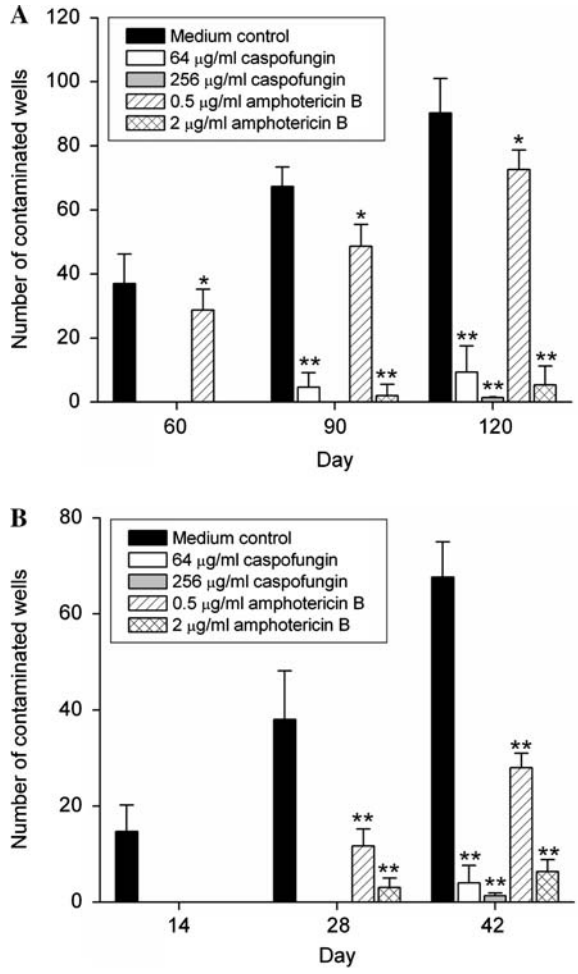

Fig. 2 Number of contaminated wells in 96-well plates with cell culture medium alone, or medium with caspofungin or amphotericin B at various days of incubation. (**A**) The plates (6 per group) were incubated at 37°C with 10% CO_2 and observed microscopically. (**B**) The plates (6 per group) were opened outside a laminar flow hood to simulate a breach in sterile technique and then incubated at 37°C with 10% CO_2. Bars are the number of contaminated wells, and brackets denote standard deviations. Asterisks denote P value significance * < 0.05; **: $P \leq 0.001$; by comparisons between medium without or with drug. These experiments were done thrice (**A**) and twice (**B**), with similar results each time

plates with 0.5 μg/ml of amphotericin B had fungal contamination by day 42. The aseptic and breach experiments were repeated thrice and twice, respectively, with similar results occurring for each iteration.

In both experiments, we observed mostly filamentous fungi including *Aspergillus* spp., *Penicillium* spp., and *Fusarium*. These fungi are ubiquitous in the

environment and generally infect cultures via an airborne route. Heating and air-conditioning systems are notorious for having high concentrations of spores. Therefore, the seasonal changes of fall and spring usually result in an increase in this type of contamination in cultures as heating or A/C systems are switched on or off. Also, particularly in the spring, the higher bioburden in the air from pollen particles can carry fungal spores into air handling systems and into labs on the clothes of lab personnel.

Caspofungin did not affect the production of antibody by hybridoma cells

There were no significant differences in antibody production by any of the hybridoma cells grown in media supplemented with caspofungin or amphotericin B. For 7C7 cells, the concentration of antibody in the supernatant was 38, 41, 42, 34, and 37 μg/ml for medium alone, medium with concentrations of 64 and 256 μg/ml of caspofungin, and medium with concentrations of 0.5 and 2 μg/ml of amphotericin B, respectively. In another experiment with 9C7 cells, the concentration of antibody in the supernatant was 42, 43, and 41 μg/ml for medium alone, medium with 64 or 256 μg/ml of caspofungin, respectively. The other hybridomas produced similar amounts of antibody in the various conditions. The values represent the average of two wells for each condition, and the experiment was repeated with similar results obtained.

Caspofungin did not alter the cytokine/chemokine production of unstimulated J774 macrophage-like cells

The cytokine/chemokine production of unstimulated and LPS-stimulated macrophages grown in the presence of caspofungin or amphotericin B was investigated. The expression of MCP-1, monocyte chemotactic protein-1, was the only immunoregulatory molecule that was significantly increased with J774 cells grown in medium alone as compared to medium in the absence of cells, which is not surprising, since it is produced by macrophage. Although there was no difference when compared J774 cells grown in medium alone and cells grown in medium supplemented with the drugs. Macrophages secreted low levels of IL-10 under all conditions. There were no differences in relative expression of the cytokines or chemokines evaluated in unstimulated J774 cells in medium alone and J774 cells in medium with caspofungin (Fig. 3). LPS-stimulated macrophages grown in the presence of both antifungal drugs showed upregulation of IL-4, IL-12, MCP-1, and TNF-α when compared to cells grown in medium alone. Furthermore, there were differences in cytokine expression when stimulated cells were exposed to caspofungin or amphotericin B. For instance, IL-4 secretion by macrophages was higher with caspofungin (31.3%) than with amphotericin B (22.4%). In contrast, stimulated macrophages showed higher levels of TNF-α when grown with amphotericin B (47.3%) than with caspofungin (25.7%). Expression of IFN-γ by LPS-stimulated J774 cells was upregulated (17.9 %) by caspofungin, but downregulated (6.3%) by amphotericin B. To support these results, concentrations of IFN-γ and TNF-α were measured by ELISA and the supernatants of LPS-stimulated J774 cells showed statistical

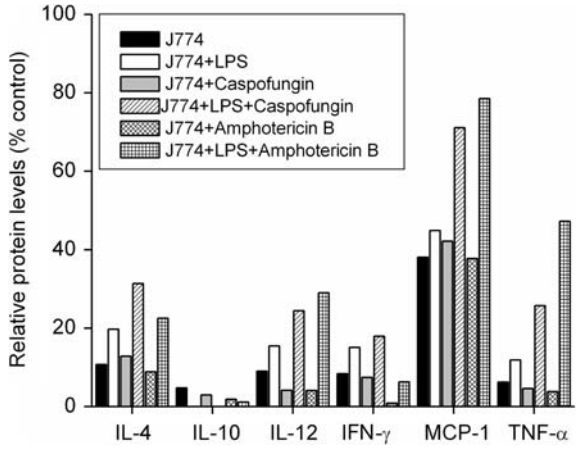

Fig. 3 Cytokine expression as measured by the RayBio mouse cytokine antibody array I kit for J774.16 cells cultured in medium with or without caspofungin (256 μg/ml) or amphotericin B (2 μg/ml) for 24 h. Controls were wells with medium alone. The relative protein levels were obtained by subtracting the background staining and normalizing to the positive controls on the same membrane. Each assay was performed in duplicate. Additionally 14 immunoregulatory molecules were also assayed by the array (http://www.raybiotech.com/map/mouse_I_map.pdf) but were of low reactivity and no differences were detected between the samples (data not shown). This experiment was done twice, with similar results each time

Table 1 Tumor necrosis factor (TNF)-α and interferon (IFN)-γ levels in supernatants of J774.16 cells (5×10^5 cells per well) cultured in medium with or without caspofungin or amphotericin B for 24 h after stimulation with LPS

Drug (μg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)
Caspofungin		
256	51	19*
512	38	11*
Amphotericin B		
2	197**	7
4	122**	2

Final cytokine expression was obtained by normalizing drug treated cells to untreated cells and LPS-stimulated untreated cells. The cytokines were measured by enzyme-linked immunosorbent assay. Results are expressed in pg/ml
$^*P < 0.001$, $^{**}P < 0.01$

differences in the production of these cytokines after incubation with caspofungin or amphotericin B (Table 1).

Discussion

Our findings suggest that caspofungin is safe and effective in reducing the incidence of fungal contamination in cell culture. Additionally, the data suggest that the drug is useful in limiting the spread of infection from one contaminated well to the other wells in a plate. We found that antibody and cytokine/chemokine production was not affected by the addition of caspofungin to the medium except when LPS was administered resulting in a modest increase in IL-4 and IFN-γ. Caspofungin and related echinocandins, such as mycofungin and anidulofungin, appear to have significantly fewer interactions with mammalian cell lines than other antifungal agents, such as amphotericin B [12], by virtue of differences in molecular structure. This phenomenon is evident in clinical use where caspofungin is remarkably well tolerated as compared to amphotericin B formulations. Additionally, we carried out the experiments with amphotericin B at 2 μg/ml, whereas the supplier (Sigma) recommends a minimal concentration of 2.5 μg/ml, a concentration at which we found substantial cellular alterations.

However, while planning cell culture studies, it is important to control for potential interactions with mammalian cells, since the echinocandins are relatively new drugs. In this regard, it is noteworthy that caspofungin have potential to interact with certain cells, as is implied by a report of an increase in histamine release from peripheral blood cells [13]. Nevertheless, our data show that the addition an echinocandin to cell culture medium may be of great benefit in reducing fungal contamination of tissues for transplantation, cell culture studies, and industrial production of biological compounds.

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