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Synergy of Caspofungin with Human Polymorphonuclear Granulocytes for Killing *Candida albicans*▽

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The influence of caspofungin on polymorphonuclear leukocyte (PMN) phagocytosis and intracellular killing of *Candida albicans* was investigated. Caspofungin, at all of the concentrations tested (2, 3.2, and 8 μg/ml), significantly increased intracellular killing by PMNs through its direct action on both yeast cells and PMNs, indicating the potential ability of caspofungin to synergize with phagocytes for candidal killing. Caspofungin may therefore constitute an effective therapeutic option for the treatment of invasive fungal infections, including those refractory to conventional treatment with azole agents.

Echinocandins, such as caspofungin, are new drugs that broaden the available therapeutic arsenal for invasive fungal infection (IFI) treatment (6, 7, 11). Caspofungin displays favorable pharmacodynamic and pharmacokinetic characteristics and has an excellent toxicological profile and antifungal activity against *Candida* spp., *Aspergillus* spp., *Histoplasma* spp., *Blastomyces* spp., and *Coccidioides* spp. (3, 7, 11, 12, 15). As the current trend in therapy requires drugs with high *in vitro* activity associated with the capacity to potentiate host defense mechanisms, especially in immunocompromised hosts (2, 19), the interaction of caspofungin with human polymorphonuclear leukocytes (PMNs) was evaluated, focusing on both the phagocytosis and intracellular killing of *Candida albicans*.

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A clinical *C. albicans* strain isolated from blood and identified by biochemical methods was subcultured on Sabouraud dextrose agar (Oxoid S.p.A., Milan, Italy) to ensure viability and purity. Yeast cultures consisted entirely of blastoconidia and had a slight tendency to differentiate into pseudohyphae during the course of the experiments.

Caspofungin acetate (Merck Sharp & Dohme Ltd., Hoddesdon, United Kingdom) was dissolved in pyrogen-free water and stored at −20°C. Antifungal susceptibility testing was performed with an inoculum of 10^3 CFU/ml, in accordance with CLSI M27-A3 (4), and an inoculum of 10^6 CFU/ml was used to perform tests with phagocytes.

PMNs were separated from lithium heparinized venous blood using Ficoll-Paque (Pharmacia S.p.A., Milan, Italy) and adjusted to 10^6 cells/ml in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) (1, 5). Viability, determined by trypan blue exclusion, was greater than 95%.

The effect of caspofungin on the phagocytosis of radiolabeled *C. albicans* ([^{3}H]uracil [specific activity, 1,270 GBq/mmol; NEN Life Science Products, Milan, Italy]) by PMNs was investigated by incubating the yeast cells (10^6 invasive fungal cells/ml) and PMNs (10^6 cells/ml) at 37°C in a shaking water bath in the presence of 2 μg/ml (MIC), 3.2 μg/ml, or 8 μg/ml caspofungin; the last two concentrations were within the range achieved clinically (8, 9). Caspofungin-free controls were included. After 30, 60, or 90 min, phagocytosis was assessed (18, 19). PMNs were centrifuged twice at 200 × *g* for 5 min to remove free blastoconidia and suspended in sterile water for 5 min; 100-μl samples were placed in scintillation fluid (Atomlight; NEN) and counted by spectrophotometry. Radioactivity

TABLE 1. Effect of caspofungin on intracellular killing of *C. albicans* by human PMNs

Time (min)	Mean SI ± SEM (% of initial fungal population killed by PMNs in absence or presence of caspofungin)			
	Control	2 μg/ml (MIC)	3.2 μg/ml[a]	8 μg/ml[a]
30	1.54 ± 0.03 (46)	1.29 ± 0.07 (71)[b]	1.28 ± 0.09 (72)[b]	1.26 ± 0.07 (74)[b]
60	1.53 ± 0.02 (47)	1.28 ± 0.11 (72)[b]	1.25 ± 0.09 (75)[b]	1.17 ± 0.06 (83)[b]
90	1.52 ± 0.03 (48)	1.25 ± 0.07 (75)[b]	1.14 ± 0.04 (86)[b]	1.17 ± 0.11 (83)[b]

[a] Concentration within the range achieved clinically.
[b] Significantly different from the control ($P < 0.01$).

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TABLE 2. Effect of preexposure of *C. albicans* or human PMNs to caspofungin for 1 h on PMN intracellular killing

Time (min)	Control	Mean SI ± SEM (% of initial fungal population killed by PMNs in absence or presence of caspofungin)					
		Preexposure of *C. albicans*			Preexposure of human PMNs		
		2 μg/ml (MIC)	3.2 μg/ml[a]	8 μg/ml[a]	2 μg/ml (MIC)	3.2 μg/ml[a]	8 μg/ml[a]
30	1.54 ± 0.03 (46)	1.31 ± 0.1 (69)[b]	1.37 ± 0.10 (63)[b]	1.37 ± 0.04 (63)[b]	1.28 ± 0.12 (72)[b]	1.31 ± 0.07 (69)[b]	1.21 ± 0.16 (79)[b]
60	1.53 ± 0.02 (47)	1.31 ± 0.06 (69)[b]	1.26 ± 0.06 (74)[b]	1.34 ± 0.04 (66)[b]	1.24 ± 0.06 (76)[b]	1.16 ± 0.12 (84)[b]	1.18 ± 0.01 (82)[b]
90	1.52 ± 0.03 (48)	1.18 ± 0.05 (82)[b]	1.22 ± 0.09 (78)[b]	1.28 ± 0.04 (72)[b]	1.18 ± 0.07 (82)[b]	1.09 ± 0.01 (91)[b]	1.06 ± 0.04 (94)[b]

[a] Concentration within the range achieved clinically.
[b] Significantly different from the controls ($P < 0.01$).

was expressed as counts per minute (cpm) per sample. The percentage of phagocytosis at a given sampling time was calculated as follows: % phagocytosis = [(cpm in PMN pellet)/(cpm in total fungal pellet)]×100 (18, 19). Intracellular killing was investigated by incubating yeast cells and PMNs (1:1 ratio) for 30 min to allow phagocytosis to proceed. The PMN-yeast cell mixtures were centrifuged at 200 × *g* for 5 min and washed to remove extracellular blastoconidia. An aliquot of PMNs was lysed by adding sterile water, and intracellular viable yeast cell counting was performed (time zero). PMNs were incubated further with 2, 3.2, or 8 μg/ml caspofungin, and at time *x* (30, 60, and 90 min), the viable counts were measured in the same way. Killing values were expressed as a survival index (SI), which was calculated by adding the number of surviving yeast cells at time zero to the number of survivors at time *x* and dividing by the number of survivors at time zero. According to this formula, if fungal killing was 100% effective, the SI would be 1 (18, 19). To differentiate between any separate effect of caspofungin on the yeast cells and PMNs, the experiments were conducted after the exposure of each of them to 2, 3.2, or 8 μg/ml caspofungin for 1 h, before they were incubated together (16, 17). After the withdrawal of caspofungin, preexposed blastoconidia were added to PMNs and blastoconidia were added to preexposed PMNs. Drug-free controls were included. Intracellular killing was determined as described above. Results were expressed as the mean ± the standard error of the mean (SEM) of 10 separate experiments, each performed in quadruplicate. Statistical evaluation of the differences between test and control results was performed by Tukey's test.

The majority of systemic antifungal drugs do not significantly influence the phagocytic activity of PMNs against *Candida* spp. (8, 13, 14). We reported that fluconazole enhances the *in vitro* killing activity of phagocytes against *C. albicans*, without improving phagocytosis (18, 19). We observed a similar pattern for caspofungin (data not shown), probably owing to the altered expression of surface molecules that link to cleavage products of complement (13). On the contrary, under the same experimental conditions, caspofungin showed a marked effect on the survival of blastoconidia, with SI values that ranged from 1.25 to 1.29, 1.14 to 1.28, and 1.17 to 1.26 for the three drug concentrations tested, compared with those for the caspofungin-free controls ($P < 0.01$; Table 1).

To determine whether the increased killing activity of caspofungin was due to its direct action on *C. albicans* or its action on phagocytes, yeast cells and PMNs were separately exposed to 2, 3.2, or 8 μg/ml caspofungin for 1 h prior to killing tests. Yeast cells pretreated with caspofungin at the MIC were killed more efficiently by the PMNs than were untreated yeast cells: SI = 1.31, 1.31, and 1.18 versus 1.54, 1.53, and 1.52 ($P < 0.01$; Table 2). Yeast killing was stimulated to about the same degree (3.2 μg/ml) or to a slightly lower degree (8 μg/ml) than that observed with caspofungin at the MIC but significantly more than that seen in the controls ($P < 0.01$; Table 2). This finding could be correlated with caspofungin's ability to unmask a virulence factor in the inner β-glucan cell wall layer, making intracellular yeast cells more susceptible to PMN lytic mechanisms (10, 21).

Pretreatment of PMNs with caspofungin at the MIC resulted in a significant enhancement of intracellular killing throughout the observation period compared with that in controls (SI = 1.28, 1.24, and 1.18 versus 1.54, 1.53, and 1.52; $P < 0.01$; Table 2). Similarly, PMNs pretreated with caspofungin at supra-MICs (3.2 and 8 μg/ml) were highly effective in clearing viable blastoconidia, compared with controls (SI = 1.31, 1.16, and 1.09 and 1.21, 1.18, and 1.06, respectively; $P < 0.01$; Table 2), thus providing indirect evidence of the drug's ability to enter phagocytes and remain available in a form that is biologically active against proliferating yeast cells. Our results differ somewhat from those obtained by van Asbeck et al. with *C. parapsilosis*, showing impairment of the innate immune mechanisms; however, these data are difficult to compare because of the differences in the experimental designs and methods used (20).

In conclusion, our findings suggest that caspofungin, at the MIC and at concentrations that are achieved clinically during antifungal therapy, displays a positive interaction with PMNs through its direct action on both yeast cells and PMNs. Caspofungin may therefore constitute an effective therapeutic option for the treatment of IFIs, including those refractory to conventional treatment with azole agents.

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