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(Article begins on next page)



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**ROLE OF CASPOFUNGIN IN RESTORING THE IMPAIRED PHAGOCYTE-
DEPENDENT INNATE IMMUNITY IN CHRONIC HAEMODIALYSIS PATIENTS
TOWARDS CANDIDA ALBICANS**

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ABSTRACT

Phagocyte-dependent cellular immunity in chronic kidney disease patients under haemodialysis treatment is frequently impaired due to the uremic state, resulting in an intrinsic susceptibility to developing invasive fungal infections (IFIs) with high mortality rates. Since synergism between phagocytic cells and antifungal drugs may be crucial for successful therapy, the aim of this study was to evaluate the effects exerted by caspofungin on the functional activities of polymorphonuclear cells (PMNs) in haemodialysed patients (HDs) towards Candida albicans compared to those of PMNs from healthy subjects (HSs).

PMNs were separated from venous blood samples of 66 HDs and 30 HSs, as controls, and measurement of phagocytic and intracellular fungicidal activities of HD-PMNs and HS-PMNs was performed in presence of caspofungin at MIC and sub-MIC concentrations. Caspofungin free-controls were also included. In the drug-free test condition, no significant difference between the phagocytic activity of HD-PMNs and HS-PMNs was detected. On the contrary, a progressive decline in the intracellular killing activity of HD-PMNs against proliferating yeasts was observed. Caspofungin at MIC and sub-MIC levels was able to improve significantly the intracellular fungicidal activity of HD-PMNs against C.albicans, restoring their functionality.

Our findings provide evidence that caspofungin exerts a synergistic effect on HD phagocyte cells against C.albicans, being able to strength the depressed intracellular killing PMN activity. These results corroborate the use of caspofungin as effective therapeutic option for IFI treatment in HD patients, in whom even a marginal influence of antifungal drugs on host response may have a relevant effect.

Keywords: PMNs, haemodialysed patients, phagocytosis, intracellular killing, *Candida albicans*, caspofungin

1. INTRODUCTION

Candida albicans infections represent a serious clinical complication in patients with chronic kidney disease (CKD) undergoing haemodialysis treatment and are associated with high mortality rate [1, 2]. The greater risk of infection in this population is caused by impaired host immunity through uraemia that interferes with T-cell and B-cell function, macrophage phagocytosis, and antigen presentation [3-6]. Systemic antifungals have been extensively used for prophylaxis and/or on an empirical basis in high-risk populations; amphotericin B deoxycholate has been so far considered the target treatment of proven invasive fungal infections (IFIs). To date, however, this treatment has been replaced with new triazoles (fluconazole and voriconazole), and echinocandins (casposfungin, micafungin and anidulafungin). Echinocandins, in fact, thanks to their high clinical efficacy, broad-spectrum activity against Candida species and several moulds, low rate of treatment-related adverse events, represent an effective treatment option in patients with refractory invasive Candida and Aspergillus infections and as empirical antifungal therapy in neutropenic patients [7]. In addition, recent evidence suggests that antifungal drugs may stimulate or alter the host immune response by mechanisms that may result in enhanced fungal clearance [8-14]. Hence, antifungal drugs with immune-enhancing properties that positively influence phagocyte activities may be crucial for resolution of fungal infections among critically ill immunocompromised patients. Recently, we have reported that casposfungin has interesting immunomodulating effects on phagocyte-mediated host responses to C.albicans, by improving the intracellular fungicidal activity of human polymorphonuclear cells (PMNs) from healthy subjects [15]. The main purpose of this study was to evaluate the potential PMN-casposfungin interaction in eradicating C.albicans, the most common life-threatening fungal pathogen in immunocompromised hosts, by studying both the phagocytic and fungicidal activities of PMNs in CKD patients under chronic haemodialysis treatment compared to those of PMNs from healthy subjects (HSs).

2. MATERIALS AND METHODS

2.1. Patients.

All patients participating in this study gave their written informed consent. Blood samples were collected from 30 healthy subjects (HSs), as controls, and from 66 haemodialysed patients (HDs), without any evidence of infection, followed at the Dialysis Center of the Ivrea Hospital, Turin, Italy: 42 males and 24 females (mean age 69.3, min 31, max 89 years). The mean time on dialysis was 52.8 months (range 1-359 months), and the causes of renal failure were as follows: chronic glomerulonephritis (8 cases), nephroangiosclerosis (18 cases), polycystic kidney disease (2 cases), diabetic nephropathy (12 cases), chronic renal failure (18 cases), kidney myeloma (2 cases), and interstitial nephritis (1 cases), other (5). The mean normalized dose of dialysis/ treatment (Kt/V single-pool = 1.44) and the protein catabolic rate (PCR = 1.15) indicated an adequate dialysis prescription and nutrition. The dialyzer membrane was modified cellulose without reuse.

2.2. Yeasts.

A clinical C.albicans strain, isolated from blood and identified by biochemical methods was subcultured on Sabouraud dextrose (SAB) 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.

2.3. Antifungal activity of caspofungin against C. albicans.

Caspofungin acetate was kindly supplied by Merck Sharp & Dohme Ltd., Hoddesdon, United Kingdom. Stock solutions of the drug were prepared into pyrogen-free distilled water and stored at -20 °C. Antifungal susceptibility testing was performed in RPMI 1640 medium (0.2% glucose) (Sigma, Milan, Italy), buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma), with an inoculum of 10^3 CFU/ml, in accordance with the CLSI guidelines M27-A3 [16], for MICs and an inoculum of 10^6 CFU/ml to perform tests with PMNs.

2.4. PMNs.

PMNs were separated from lithium heparinized venous blood using Ficoll[®]Paque (Pharmacia S.p.A., Milan, Italy), as previously described in detail [15, 17-21]. PMN viability, determined by trypan blue exclusion, was greater than 95%.

2.5. Influence of caspofungin on phagocytosis and intracellular killing.

The effect of caspofungin on the phagocytosis of radiolabelled *C.albicans* [³H-uracil (specific activity: 1,270 GBq/mmol; NEN Life Science Products, Milan, Italy)] by PMNs was investigated by incubating yeasts (10⁶CFU/ml) and PMNs (10⁶cells/ml) at 37°C in a shaking water bath for periods of 30, 60 or 90 min in the presence of 1xMIC, ½xMIC and ¼xMIC caspofungin. Caspofungin-free controls were included. After 30, 60 or 90 min, phagocytosis was assessed. PMNs were centrifuged twice at 200 x g for 5 min to remove free blastoconidia and suspended in sterile water for 5 min; 100µl samples were placed in scintillation fluid (Atomlight, NEN) and counted by spectrophotometry. Radioactivity 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 (15). Intracellular killing was investigated by incubating yeast cells and PMNs (1:1 ratio) for 30 min to allow phagocytosis to proceed. The PMN-yeast cells mixtures were centrifuged at 200 x 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 1xMIC, ½xMIC and ¼xMIC of 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 (15).

2.6. Statistical analysis.

Statistical analysis was applied using the Graphpad Prism version 3.00 for Windows (Graphpad Software, San Diego, Calif., USA). Results were expressed as the means and standard error of the

means (SEMs) for 10 separate experiments, each performed in quadruplicate. Evaluation of differences between test and control results was performed by using Student's unpaired t-test. *P*-values < 0.05 were considered statistically significant.

3. RESULTS

MICs of caspofungin for C.albicans strain were 0.5 µg/ml with an inoculum of 10³ CFU/ml and 2 µg/ml with an inoculum of 10⁶ CFU/ml. Both phagocytosis and intracellular killing of PMNs from HS and HD groups against C.albicans were evaluated in presence of 2 µg/ml of the drug. In all experiments, the viability of PMNs remained unchanged throughout. In the drug-free test condition, there was no significant difference between the phagocytic activities of HD and HS-PMNs during the 90 min incubation time (Table 1). On the contrary, HD-PMNs were less effective in eradicating proliferating yeasts than HS-PMNs, with reduced intracellular fungicidal activity. In fact, blastoconidia were only partially killed by HD-PMNs, with SI values of 1.70, 1.75 and 1.73 at 30-60-90 min of incubation, whereas a significantly higher fungicidal effect was detected for HS-PMNs at all incubation times, with SI values of 1.54, 1.53 and 1.52, respectively (Table 1). Caspofungin at 1xMIC concentration did not influence significantly the phagocytic activity of PMNs in HD or HS groups, as we detected phagocytosis rates similar to drug-free HD-controls and to those observed in HS-PMNs (Table 2). Conversely, caspofungin at 1xMIC concentration, added to PMNs after phagocytosis had occurred, significantly improved the intracellular fungicidal activity of PMNs in both groups, especially after 60 and 90 min of incubation: in fact, SIs ranged from 1.47 to 1.30 compared to SIs of drug-free HD-controls, ranging from 1.75 to 1.73 (Table 2). Similar results were also obtained when HD-PMNs were exposed to subinhibitory caspofungin concentrations: at 90 min incubation time in the presence of either ½xMIC or ¼xMIC the ingested yeast cells were 74.2% and 73.8%; with SI values of 1.32 and 1.34, respectively.

4. DISCUSSION

Literature data support the notion that uraemia is associated with an acquired immune deficiency which involves both cellular and humoral immunity, with the degree of impairment related to the duration of the uremic state [3-6]. According to these studies, bacterial phagocytosis and intracellular killing are impaired in PMNs from uraemic patients, as a consequence of a reduced ability to generate superoxide in response to stimuli as well as impaired chemotaxis and degranulation. However, the factors related to the whole PMN dysfunction are still not completely understood and have been ascribed to malnutrition, iron overload, uraemic toxins, elevated levels of intracellular calcium, zinc deficiency and dialysis per se [17, 22].

Recently, we have reported that caspofungin, belonging to the echinocandin drug class, as an adjunct to its direct fungicidal activity, exerts an indirect immune-modulating effect on HS-PMNs through enhancement of intracellular killing against C.albicans without affecting the phagocytic activity [15]. Other investigators have shown a positive interaction between host phagocytic cells and echinocandins, including caspofungin, that resulted in enhanced killing of Candida and Aspergillus species [8-13, 23]. To further highlight the potential ability of caspofungin to synergize for fungal killing with PMNs even in immunocompromised hosts, we evaluated the PMN antifungal functions and the potential PMN-caspofungin interaction in HD patients against C.albicans.

Based on our in vitro results, PMNs from haemodialysis patients were able to phagocytose yeast cells, with a phagocytosis pattern quite similar to that detected in HS-PMNs (Table 1). On the other hand, a progressive decline in the fungicidal activity of PMNs from HD patients with CKD was confirmed [24]. In fact, as reported in Table 1, the killing function was significantly lower in the HD group than in the HS control-group (30, 25 and 27% of killing in HD-PMNs vs 46, 47 and 48 % of killing in HS-PMNs), thus implying that immune deficits in these severely ill patients are associated with a compromised host-immune ability to clear efficiently invading yeasts, even though they have been successfully phagocytosed.

As the majority of systemically acting antifungal drugs, caspofungin did not significantly improve the phagocytic activity. Conversely, the fungicidal activity of PMNs from HD patients was

significantly potentiated by caspofungin, especially after 60 and 90 min of incubation, in comparison with caspofungin-free controls, as we detected killing percentages of 53 and 70% in caspofungin treated HD-PMNs vs killing percentages of 25 and 27% in drug-untreated HD-PMNs (Table 2). The delay in the fungicidal effects of caspofungin exposed HD-PMNs on C.albicans may be related to the underlying inability of phagocyte killing mechanisms to act initially, which is effectively restored by subsequent PMN-caspofungin combined action (Table 2). A similar pattern was also detected when HD-PMNs were exposed to subinhibitory caspofungin concentrations, indicating absence of a drug dose-dependent effect.

The increased killing activity observed in HD-PMNs after exposure to MIC and sub-MIC caspofungin concentrations may be related to a drug action on both C.albicans and phagocytes. In fact, as recently reported by literature data, echinocandins disrupt the external layer of the fungal cell wall, thus allowing immune cells access to immunoreactive epitopes of beta-glucan, a major component of the cell wall of Candida species, with subsequent enhancement in fungal recognition (13,14). Furthermore, the hypothesis of a tight fungicidal co-operation between PMNs and caspofungin is corroborated from our previous studies on PMN functional activities in healthy subjects (15), where it emerged that the same C.albicans strain, pre-treated with 1xMIC caspofungin for 1 hour prior to killing tests, was killed more efficiently by HS-PMNs than was untreated control. Similarly, in the same study, PMNs pre-treated with 1xMIC caspofungin were highly effective in clearing viable yeast cells compared with untreated controls, thus providing indirect evidence of the drug's ability to enter phagocytes and remain available in a biologically active form within phagocytes (15).

These findings on caspofungin activity in HD-PMNs are in agreement with our previous work on PMN phagocytic activity towards C.albicans in renal transplant recipients (RTRs). In fact, we observed a similar enhancement of the PMN fungicidal activity with caspofungin, being able to synergize with PMNs from RTRs against yeast intracellular growth, without alteration in phagocyte uptake [25].

In conclusion, our current data provide evidence that caspofungin is able to restore the depressed intracellular killing of PMNs in CKD patients on haemodialysis treatment, through a synergistic effect with PMNs towards C.albicans and may constitute effective therapeutic option for IFI treatment in patients with altered phagocyte-dependent innate immunity. As the therapeutic efficacy of antifungal drugs appears to be limited without the help of host immune reactivity, this synergistic drug effect on host phagocyte cells could be relevant to patients receiving caspofungin for either prophylaxis or therapeutic purposes. Further ex vivo studies on PMNs of patients receiving the drug will be needed to elucidate the clinical relevance of these in vitro results. In fact, antifungal efficacy with the ability to restore or enhance the host immune system could provide a new therapeutic regimen for the immunocompromised patient, in whom even a marginal influence of antifungal drugs on host response may have a significant effect, and may represent an important direction for future drug-related research.

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Ethical approval: Not required

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TABLES

Table 1. Functional activity of PMNs from healthy subjects and chronic haemodialysis patients: phagocytosis and intracellular killing against C. albicans.

Time	Mean % phagocytosis \pm SEM	Survival Index (SI) \pm SEM (%)*
Healthy subjects		
30 min	77.1 \pm 0.67	1.54 \pm 0.03 (46%)
60 min	75.8 \pm 0.11	1.53 \pm 0.02 (47%)
90 min	75.1 \pm 0.32	1.52 \pm 0.03 (48%)
Chronic haemodialysis patients		
30 min	73.2 ^a \pm 0.06	1.70 ^b \pm 0.03 (30%)
60 min	75.6 ^a \pm 0.03	1.75 ^c \pm 0.02 (25%)
90 min	74.5 ^a \pm 0.04	1.73 ^c \pm 0.05 (27%)

* (%) Percentages of yeasts killed by PMNs.

^a Not significantly different from healthy subject controls ($P > 0.05$).

^b Significantly different from healthy subject controls ($P = 0.01$).

^c Significantly different from healthy subject controls ($P < 0.001$).

Table 2. Influence of caspofungin (1xMIC) on phagocytosis and intracellular killing of PMNs from healthy subjects and chronic haemodialysis patients against *C. albicans*.

Time	Mean % phagocytosis \pm SEM		Survival Index (SI) \pm SEM (%) [*]	
	Controls	Caspofungin (2 μ g/ml)	Controls	Caspofungin (2 μ g/ml)
Healthy subjects				
30 min	77.1 \pm 0.67	77.2 ^a \pm 0.76	1.54 \pm 0.03 (46%)	1.29 ^b \pm 0.07 (71%)
60 min	75.8 \pm 0.11	75.1 ^a \pm 0.11	1.53 \pm 0.02 (47%)	1.28 ^b \pm 0.11 (72%)
90 min	75.1 \pm 0.32	75.9 ^a \pm 0.09	1.52 \pm 0.03 (48%)	1.25 ^b \pm 0.07 (75%)
Chronic haemodialysis patients				
30 min	73.2 \pm 0.06	73.5 ^a \pm 0.02	1.70 \pm 0.03 (30%)	1.63 ^a \pm 0.06 (37%)
60 min	75.6 \pm 0.03	75.2 ^a \pm 0.15	1.75 \pm 0.02 (25%)	1.47 ^b \pm 0.03 (53%)
90 min	74.5 \pm 0.04	74.8 ^a \pm 0.31	1.73 \pm 0.05 (27%)	1.30 ^b \pm 0.03 (70%)

^{*}(%) Percentages of yeasts killed by PMNs in absence and in presence of the antifungal drug.

^a Not significantly different from controls without drug ($P > 0.05$).

^b Significantly different from controls without drug ($P < 0.001$).