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Analyses of phagocytosis, evoked oxidative burst, and killing of black yeasts by human neutrophils: A tool for estimating their pathogenicity?

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The pathogenicity of several dematiaceous yeasts that have, to date, rarely been isolated in humans remains unclear. Because professional phagocytes are prominent in lesions caused by dematiaceous fungi, we address this issue by comparing phagocytosis, evoked oxidative burst and killing by human neutrophils of different black yeasts *in vitro*. Whereas phagocytosis of all black yeasts tested and evoked oxidative burst yielded comparable results, in contrast, the degree of killing differed significantly after 5 h. Thereby, two groups could be identified; one in which strains are killed at high rates, for example, *Hortaea werneckii* (81 ± 11.6%), *Exophiala castellanii* (96 ± 8.6%), *Phaeoannellomyces elegans* (93 ± 9.7%), *Phaeococcomyces exophialae* (87 ± 8.7%), and the other in which strains are killed to a lesser degree, for example, *Exophiala dermatitidis* (ATCC 34100) (61 ± 9.5%), *E. dermatitidis* (CBS 207.35) (66 ± 7.5%), *E. jeanselmei* (50 ± 10.5%), *E. mesophila* (63 ± 11.6%), *E. bergeri* (63 ± 9.1%), and *E. spinifera* (57 ± 9.6%). Non-pigmented yeasts were killed at levels comparable with those at which the white mutant strain of *E. dermatitidis* (ATCC 44504) was killed (95 ± 7.5%); the yeast strains tested were *Candida albicans* (DSM 11943) (95 ± 4.0% killing) and *Saccharomyces cerevisiae* (DSM 1333) (95 ± 10.3%). Comparison of killing rates with the observed pathogenicity of the melanized species suggests that low killing rates might indicate or even predict a high degree of invasiveness. Although previous experiments revealed that melanization conferred killing resistance on *E. dermatitidis*, the differences in killing rates of other dematious fungi suggest that melanization of the cell wall is in itself insufficient to confer virulence.

Keywords dematiaceous yeasts, human neutrophils, killing, melanin

Introduction

Dematiaceous fungi that multiply by budding in certain stages of their development or under certain environmental conditions are generally referred to as 'black yeasts'. Diseases caused by these organisms comprise mycetomas, chromoblastomycosis and more often phaeohyphomycosis [1]. Mycetomas are chronic infections characterized by deep lesions with black granules, tumefaction and draining sinus tracts. The only black yeast species involved in causing this disease is *Exophiala jeanselmei*. Chromoblastomycosis, which can also be caused by *Exophiala spinifera*, is nowadays defined as a distinct clinical entity. It is characterized by long-lasting chronic infections with muriform elements. These

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elements are large thick-walled fungal cells located in the tissue; they are often referred to as sclerotic bodies [2]. In phaeohyphomycosis, in contrast, dark-walled hyphal elements are predominant in the tissue. Phaeohyphomycosis occurs as: (i) superficial infections limited to the stratum corneum and not eliciting tissue response, i.e., tinea nigra and corneal infections; (ii) cutaneous infections with invasion of keratinized tissue; (iii) subcutaneous infections frequently with cyst formation; and (iv) visceral and systemic infections [3]. Tinea nigra, a superficial infection of the stratum corneum of the palm or sole, is a common disease in tropical and subtropical climates clinically appearing as a well-circumscribed blackish macula. It can be caused by *Hortaea werneckii*, *Exophiala castellanii* or *Stenella araguata*. The species causing phaeohyphomycosis mainly belong to the genus *Exophiala*, or to its synanamorphic genera *Phaeoannellomyces* and *Phaeococcomyces*. *E. dermatitidis* has been described as an important agent of systemic phaeohyphomycosis with a marked tendency to invade the central nervous system. The latter manifestation is associated with a high fatality rate, 48% [4,5].

Although some dematiaceous fungi have been clearly shown to cause superficial or invasive diseases, most have, to date, only rarely been isolated in humans or animals, and therefore their true pathogenic potential has remained unclear. Because one of the predominant host-cell types in dematiaceous lesions consists of professional phagocytes, that is, macrophages and neutrophils [6], the capacity of these cells to kill pathogens could be considered as a test for potential invasiveness. Therefore, this study used a recently developed *in vitro* assay [7] to assess phagocytosis, evoked oxidative burst and killing by human neutrophils. The results have given us the potential to estimate pathogenicity of so far rarely isolated black yeasts.

Materials and methods

Strains and culture conditions

The origins and characteristics of strains used in this study are shown in Table 1. Strains derived from the taxonomic type matêrial of species are indicated by a superscript T, whereas those connected with neotypes are noted as NT. To obtain yeast-like growth, strains were cultured in different media. The basic culture medium contained 50 g yeast extract (Difco Laboratories, Detroit, MI), 10 g D-glucose-monohydrate (Merck AG, Darmstadt, Germany), 17.26 g 3-(*N*-morpholino)-propanesulfonic acid sodium salt (MOPS) (Sigma, München, Germany), 6.7 g yeast nitrogen base (Difco), and 1.91 g MOPS acid (Sigma) made up to 1 l with distilled water (pH 7.4 at 25°C). The basic culture medium was used for *E. bergeri* (incubated 5–6 days), *E. dermatitidis* and its melanin-deficient mutant strain ATCC 44504 (3–4 days), *E. spinifera* (4–5 days); *Candida albicans* (1–2 days) and *Saccharomyces cerevisiae* (1–2 days). To aid the growth of *E. jeanselmei* (incubated 8–10 days), *E. mesophila* (6–8 days) and *H. werneckii* (2–3 days), the basic medium was supplemented with 0.93 g l^{-1} ethylenediaminetetraacetic acid (EDTA) sodium salt. For *Phaeoannellomyces elegans* (incubated 67 days), the pH was adjusted to 3.5 by replacing MOPS with 3.46 g l^{-1} disodium hydrogen phosphate and 18.92 g l^{-1} citric acid monohydrate (all Merck AG). *Phaeococcomyces exophialae* (incubated 10–11 days) and *E. castellanii* (21–28 days) were cultured in Sabouraud broth (pH 6.0 at 25°C) (Oxoid, Wesel, Germany) containing 4% (w/v) glucose. Finally, the culture broth was filtered through a gauze bandage (12 layers; HGV-Verbandstoffe, Lindhorst, Germany) to remove hyphal elements before measurements were taken.

Measuring phagocytosis and oxidative burst using flow cytometry

To assess phagocytosis, washed (5 ml sterile 0.9 % w/v NaCl), sedimented (3000 rpm at 4°C for 5 min), and sonicated (2 min, half-maximal energy; Transsonic 460/H, ELMA, Singen, Germany) yeast cells from 5 ml broth were incubated with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, pentaacetoxymethyl ester (BCECF/AM; Roche Diagnostics, Mannheim, Germany; final concentration 1 mol l^{-1}) for 30 min at 37°C in 1 ml phosphate-buffered saline (PBS) as described previously [7]. Non-fluorescent BCECF/AM diffuses into the yeast cells and is cleaved by cytoplasmic esterases removing the pentaacetoxymethyl ester and yielding the fluorescent, membrane-impermeable BCECF. This BCECF remains trapped in viable cells emitting a green fluorescent signal upon excitation by blue light (488 nm). We incubated 5×10^6 of such labelled yeast cells at 37°C in a thermomixer (Eppendorf, Hamburg, Germany) at 1000 rpm along with 1 ml heparinized (10 IE, approximately corresponding to 5 USP ml^{-1}) whole blood obtained from healthy voluntary donors ($n = 30$, 18 female, 12 male; age 25–50 years) for a maximum of 120 min. All the assays were performed with standardized yeast cell-to-granulocyte ratios varying slightly from 1.5 to 2.5. Also, each yeast strain was repeatedly tested ($n = 8$) using blood from different donors to eliminate misinterpretation due to donor-specific effects. At 0, 10, 30 and 60 min, 100 µl were removed and immediately mixed with 2 ml ice-cold lysis buffer (Becton Dickinson, Heidelberg, Germany) to lyse the erythrocytes. Within 2 h leukocytes and yeast cells

Table 1 Characteristics of strains tested

Strains used	Obtained as	Isolated from	Place of origin	Reported infections or infectivity of species or mutants types
Candida albicans	DSM 11943	Human (Pfizer Ltd., Y01.09)	nd	Superficial and systemic infections especially in immunocompromised patients
Exophiala bergeri^{T}	CBS 526.76	Subepidermal cyst of human [13]	Memphis, USA	Subcutaneous (as *Sporotrichum gougeroti*) [21,22]
E. castellanii^{NT}	CBS 158.58	Tinea nigra [14]	Sri Lanka	Tinea nigra, subcutaneous [23,24]
E. dermatitidis^{T}	CBS 207.35	Facial phaeohyphomycosis [25]	Japan	Keratomycosis, phaeohyphomycosis, systemic neurotropic mycosis in Asia, subclinical colonization of lungs of cystic fibrosis patients, chronic otitis externa, onychomycosis [4,2638]
E. dermatitidis	ATCC 34100	Patient's lung [39]	Japan (?)	Keratomycosis, phaeohyphomycosis, systemic neurotropic mycosis in Asia, subclinical colonization of lungs of cystic fibrosis patients, chronic otitis externa, onychomycosis [4,2638]
E. dermatitidis	ATCC 44504	Spontaneous albino mutant of ATCC 34100 (Mel^{3-}) [39]		In animal experiments has lower virulence than wild-type strain [40]
E. jeanselmei^{T}	ATCC 34123	Mycetoma of foot [23]	France	Phaeohyphomycosis, eumycotic mycetoma, dental granuloma, arthritis, endocarditis, keratitis, endophthalmitis, systemic infection [3,10]
E. mesophila^{T}	CBS 402.95	Silicon seal [11]	Germany	Not yet observed [11]
E. spinifera^{T}	CBS 107.67	Nasal granuloma [41]	USA	Disseminated infections, chromoblastomycosis, verrucous skin lesions [4249]
Hortaea werneckii^{NT}	CBS 107.67	Tinea nigra [14]	Portugal	Tinea nigra [15,50,51]
Phaeoannellomyces elegans^{T}	UTMB 1286	Phaeohyphomycosis (toenail) [52]	Canada	Phaeohyphomycosis? [16,52]
Phaeococcomyces exophialae^{T}	CBS 668.76	Straw (burrow of *Dasypus septemcintus*) [8]	Uruguay	? [52]
Saccharomyces cerevisiae	DSM 1333	Distillery yeast	Nd, control strain	Extremely rare cause of infections

nd, no data.
T, strain ex type.
NT, strain ex neotype.

were harvested by centrifugation (10 min, 4°C, 1300 rpm; Beckman GS-6R centrifuge), washed twice in ice-cold PBS, resuspended in 500 µl PBS and analysed by flow cytometry.

Flow-cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson) using the Cellquest software (Becton Dickinson) to evaluate the data. Instrument settings, linear parameters for forward scatter (FSC) and side scatter (SSC), as well as logarithmic parameters for green fluorescence (FL1) and orange–red fluorescence signal (FL2) received by the photomultiplier tube, were all set at their best test performance levels, which were judged to be the same as those used previously for experiments with *E. dermatitidis* and its melanin-deficient mutants [7]. Binding of the yeast cells by the neutrophils was analysed as follows [7]. Neutrophils were selectively analysed by gating them according to their relative size and granularity. The association of neutrophils with the BCECF-labelled yeast cells was expressed as an increase of the green fluorescence of the neutrophils. The percentage of neutrophils exhibiting a green fluorescence was determined using the quadrant statistics in Cellquest. The fluorescence cut-off was set prior to addition of yeast cells to categorize 99% of the neutrophils as being located within the non-fluorescent quadrant.

To determine the production of H_2O_2, as well as reactive oxidative intermediates produced through the release of ions such as O^{2-} or OH^{-} by neutrophils during the respiratory burst, unlabelled yeast cells were incubated in heparinized blood under conditions identical to those described above but in the presence of dihydrorhodamine (DHR; Molecular Probes, Eugene, OR). DHR was added to a final concentration of 10 mg l^{-1} as described previously [7]. DHR permeates freely, localizes in the mitochondria of neutrophils and,

after oxidation by H_2O_2 and O^{2-} to rhodamine 123 during the respiratory burst, emits a bright green fluorescent signal upon excitation by blue light (488 nm). Samples were prepared and analysed by flow cytometry as described for the phagocytosis assay. Oxidative burst of the neutrophils induced by unstained yeast cells in the presence of DHR was expressed as an increase of the green fluorescence of the neutrophils. Flow-cytometric instruments settings, linear parameters for FSC and SSC, logarithmic parameters for FL1 and FL2 for evaluation of data of this assay were the same as used previously [7].

Microscopy

To ensure intracellular location of the yeast cells associated with the neutrophils, representative samples used for determination in flow cytometry were additionally examined by epifluorescence interference contrast microscopy (Leitz DM RB; Leica, Wetzlar, Germany) as described previously [7].

Killing assay

To quantify the killing of the strains tested by human neutrophils, culturing was performed as outlined in 'Strains and culture conditions' above. The harvested and washed yeast cells were diluted in PBS to 10^5 cfu ml^{-1}. This inoculum did not increase or diminish during the time of assay performance. Six hundred microlitres of diluted cells was added to 1800 µl heparinized blood and rotated end-over-end at 37°C at 8 rpm (rotator type 3025, G. F. L., Burgwedel, Germany) for 5 h. For each test isolate, any adherence of cells to the test tubes in a manner that could alter inoculum levels was excluded by light-microscopy checking of the walls of test tubes incubated with yeast inoculum for 5 h in leukocyte-free blood (data not shown). Initially and after 10 min, and 1, 2, 3, 4 and 5 h of rotation, 100 µl of a mixture of yeast cells and blood were added to 900 µl of NaCl (0.9% v/v). Viable counts (cfu) were determined in quadruplicate by plating 100 µl samples from each time point onto Sabouraud dextrose agar (SDA). Mean cfu values were calculated and the percentage of killed yeast cells was deduced; values are given as these percentages. As with the phagocytosis and burst assays, each isolate was repeatedly tested ($n = 8$) using blood from different donors to exclude donor-specific effects.

To estimate potential loss of viability for species with growth optima below 37°C, namely, *E. castellanii*, *E. mesophila*, *H. werneckii*, *P. elegans* and *P. exophialae*, cell numbers were estimated at time 0 and after 5 h of incubation in serum at 37°C using an inoculum of 10^5 cells ml^{-1}.

Statistics

Statistical analysis was performed by the non-parametric Mann-Whitney *U*-test for unpaired samples. Probability values ≤ 0.05 were considered statistically significant.

Results

Phagocytosis and oxidative burst

Flow cytometric evaluation distinguished the different yeast cells from blood cells (granulocytes, lymphocytes, monocytes). As shown recently for *E. dermatitidis* [7], and for the different black yeasts tested here, the resulting green fluorescence was strong enough to be detected reliably by flow cytometry (at 10^3 relative fluorescence units) although not affecting cell viability (data not shown). In the initial measurement, only a low percentage of granulocytes ($\leq 10\%$) was found to be activated, as judged by their green fluorescence in the respiratory burst assay (data not shown).

After 1 h, phagocytosis rates were comparable for all species analysed, and ranged from 74% (with *E. dermatitidis* CBS 207.35 and melanin-deficient mutant strain *E. dermatitidis* ATCC 44504) to 90.5% (with *E. spinifera*). Over a co-incubation times of 0, 30 and 60 min, the phagocytosis rate did not differ significantly among the species tested.

All yeasts tested evoked oxidative burst. After 30 min, the level of fluorescing neutrophils ranged from 67% (with *P. elegans*) to 94% (with *E. spinifera*). For all strains tested, oxidative burst levels were found to increase in parallel with the phagocytosis rate. Results are displayed in Figures 1 and 2.

Microscopy

Microscopic evaluation revealed that after 30 min, between 60 and 98% of the yeast cells were located inside the neutrophils. After 60 min, > 95% of the cells had been ingested by neutrophils in all strains tested. These results are comparable with those obtained previously [7]. For all strains tested, microscopic evaluation throughout the experiment demonstrated no occurrence of distinctive morphological features, for example, germ tube formation. There was a uniform appearance of neutrophils each of which contained two to six yeast cells.

Killing assay

Mean cfu counts for the repeatedly performed killing assays (for each strain $n = 8$) are shown in Figure 3. No temperature-related loss of viability could be seen for species with growth optimum below 37°C (data not

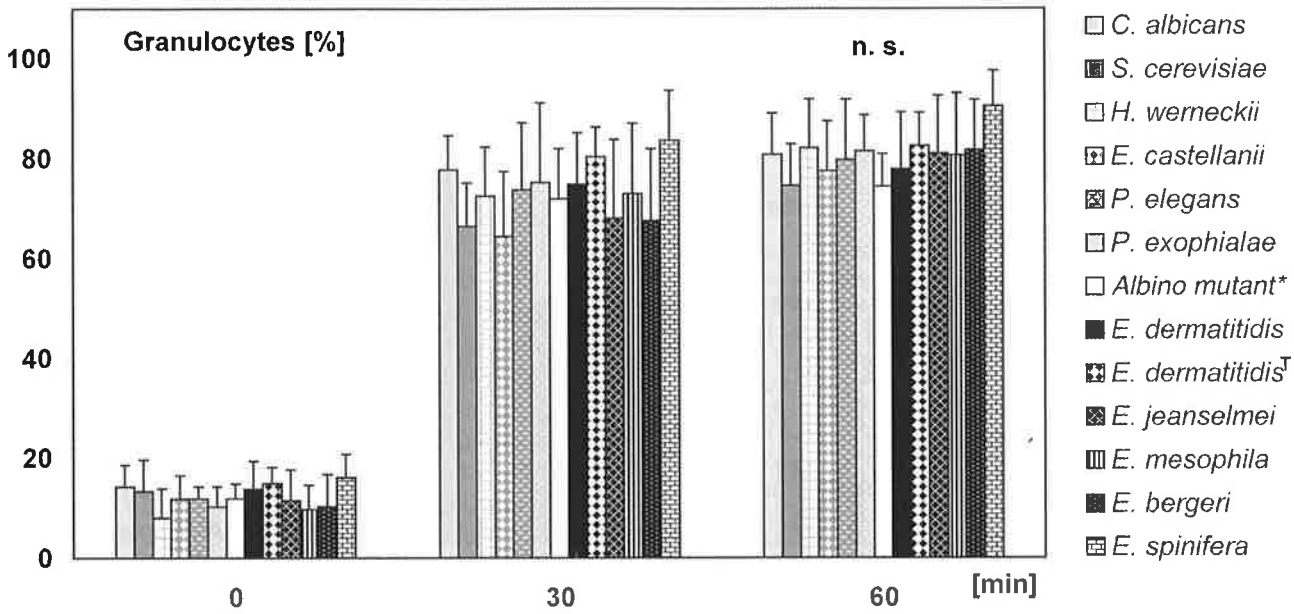

Fig. 1 Kinetics of the binding of black yeasts and non-melanized controls (each $n = 8$) to human neutrophils as determined by flow cytometry. The subsequent percentage increase of neutrophils associated with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF)-stained yeast cells is shown (mean values ± SD); ns, differences not significant, $P \leq 0.05$).

shown). Two groups within the black yeasts could be identified. After 5 h of co-incubation, group I members were found to be killed to a high level (mean values: 81–96%), and group II members to a low degree (mean values: 50–66%). Group I comprised *H. werneckii* (killing level 81 ± 11.6%), *E. castellanii* (96 ± 8.6%), *P. elegans* (93 ± 9.7%), and *P. exophialae* (87 ± 8.7%). Group II comprised *E. dermatitidis* (ATCC 34100) (61 ± 9.5%), *E. dermatitidis*^{T} (CBS 207.35) (66 ± 7.5%), *E. jeanselmei* (50 ± 10.5%), *E. mesophila* (63 ± 11.6%), *E. bergeri* (63 ± 9.1%), and *E. spinifera* (57.1 ± 9.6%). No differences in killing rate were observed for the non-pigmented yeasts, *C. albicans* (95.3 ± 4.0%) and *S. cerevisiae* (95.0 ± 10.3%) and the

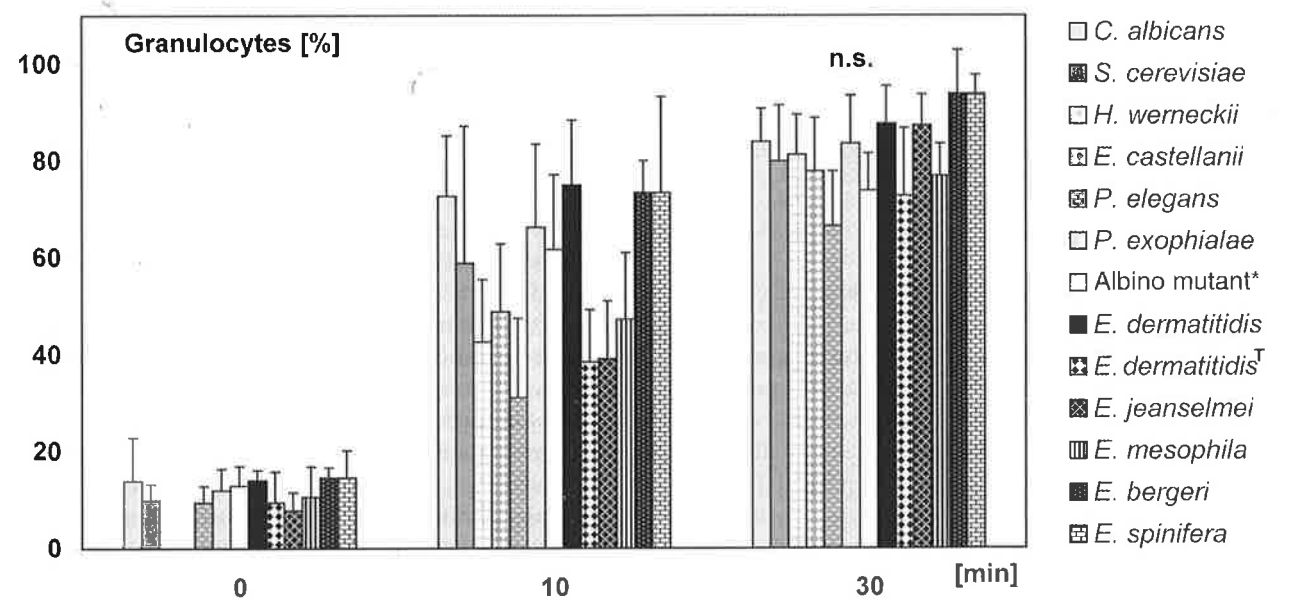

Fig. 2 Kinetics of the oxidative burst of neutrophils evoked by black yeasts and non-melanized controls (each $n = 8$) as determined by flow cytometry. The percentages of neutrophils exhibiting a green fluorescence after co-incubation with non-labelled yeast cells in the presence of dihydrorhodamin (DHR) (mean values ± SD; ns, differences not significant, $P \leq 0.05$) are shown. In case of *H. werneckii* and *E. castellanii*, 0 min standard deviation could not be illustrated graphically (<1%).

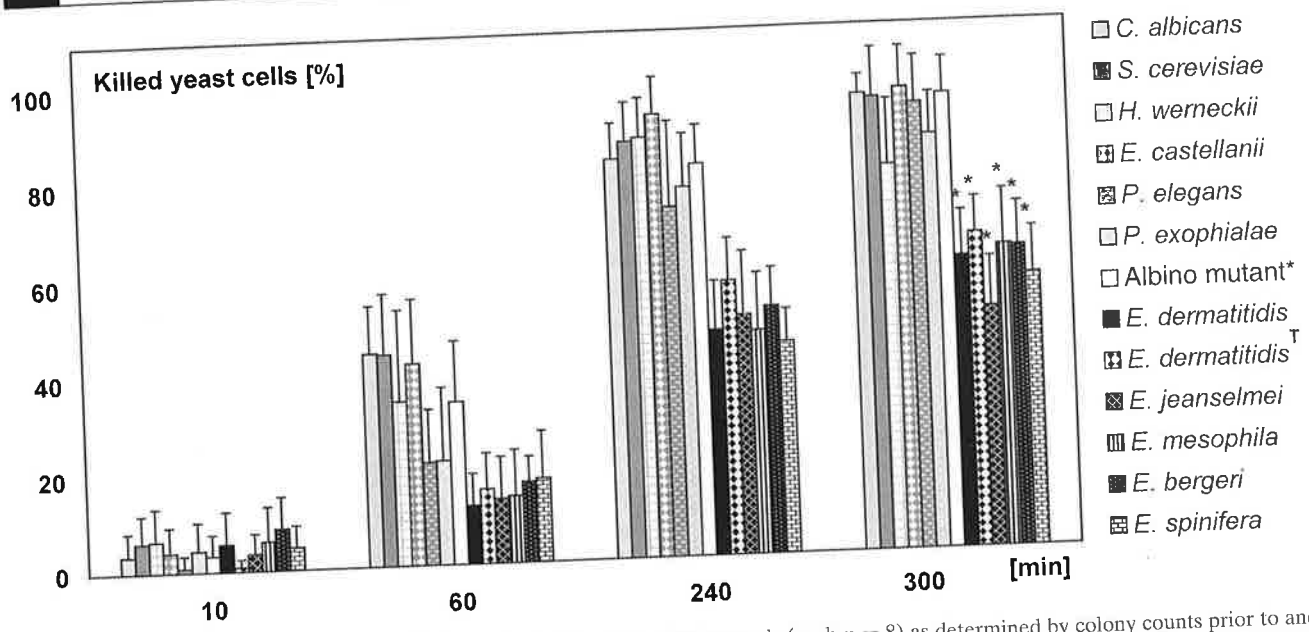

Fig. 3 Percentage of killing of different black yeasts and non-melanized controls (each $n = 8$) as determined by colony counts prior to and after incubation in heparinized human blood. The killing is displayed as a percentage of the decrease in the number of cfu during the incubation times indicated. The mean values ± SD are shown; *significantly different from the 5 h value of *Hortaea werneckii* ($P \leq 0.02$).

white melanin-deficient mutant strain of *E. dermatitidis* (ATCC 44504) (95 ± 7.5%) after 5 h [7].

Statistical comparisons

Statistical evaluation of phagocytosis (60 min) and oxidative burst (30 min) did not reveal significant differences between the two fungal groups distinguished in the killing assay ($P \leq 0.05$). This is in striking contrast to highly significant difference ($P = 0.001$) obtained in the killing assay itself, even when the group II strain with the highest mean killing rate (*E. dermatitidis*^{T}, CBS 207.35; 66 ± 7.5%) was compared with the group I strain showing the lowest mean killing rate (*H. werneckii*, 81 ± 11.6%).

Discussion

Neutrophils are the predominant phagocytotic cells in lesions caused by dematiaceous fungi, and their successful killing of yeast cells helps to prevent further invasive growth of infecting fungi. Therefore, low killing rates for a given species can be taken as a predictor of potential invasiveness and higher overall pathogenicity for humans. Recently, we have developed a method for the measurement of these phagocyte functions. To investigate the effect of melanin and carotinoids from *E. dermatitidis* on phagocyte function we have used flow cytometry in combination with a killing bioassay [7] to compare phagocytosis rates for *E. dermatitidis* and its albino mutants after exposure to human neutrophils. Including these strains in this study our previous results could be confirmed, thereby substantiating the reproducibility of the developed phago/burst and killing assay.

Our study showed that the black yeasts tested could be categorized as group I with a predicted low invasive potential, and group II with a predicted high invasive potential. For relatively frequently encountered black yeasts, these categories corresponded for the most part with the actual pathogenicity as observed to date. For the more rarely encountered black yeasts, the results of this study may enable a preliminary estimation of pathogenicity. For *E. dermatitidis* and *E. jeanselmei,* categorization as group II conforms with our expectations because these yeasts are known to cause lethal cerebral infections [8]. The reported ability of *E. dermatitidis* to invade via hematogenous dissemination also substantiates the potential invasiveness of this species [9]. *E. spinifera* has also been reported to cause severe human infections. Owing to high case fatality rate of these mostly progressive systemic infections [10], the grouping of this species in this study with other highly pathogenic species seems to be congruous. In contrast, the grouping of *E. mesophila* with relatively pathogenic species awaits further investigation. This recently described species has so far only been isolated from the environment [11, de Hoog, unpublished observation]. The situation is similar with *E. bergeri* [12], which, although grouped with the more pathogenic species, has been known to cause cutaneous and subcutaneous infections, but as yet no described disseminated infection [13].

E. castellanii and *H. werneckii* cause only a superficial infection, tinea nigra [14,15], which is consistent with

their designation as members of group I. In the cases of expected low-pathogenicity yeasts *P. exophialae* and *P. elegans,* which are synanamorphs of an as yet unknown *Exophiala* species [12], only one infection (ascribed to *P. elegans*) has been described, and its symptoms include a chronic, painful nodule on the palmar surface [16]. Further details of reported infections for the species studied are given in Table 1.

All black fungi possess melanin deposited in or on the cell wall. The melanin is known to absorb light and heat energy owing to its numerous free carboxyl groups. This accounts for many of the protective as well as photosensitizing properties of melanin [17]. The function of melanin as a virulence factor in *Exophiala* has clearly be shown by comparing a melanized *E. dermatitidis* strain with its corresponding isogenic albino mutant in a bioassay using whole human blood [18]. This study found that the presence of melanin conferred lower killing rates on *E. dermatitidis* [18]. In a mouse acute-infection model [18], an albino (melanin-deficient) mutant strain of *E. dermatitidis* (ATCC 44504) was confirmed to have lowered resistance to killing and to be less virulent. For all melanized yeasts analysed here, we would therefore have expected a relatively high resistance to killing by human neutrophils and resulting high survival rates, particularly as evoked oxidative burst was comparable for all black yeasts studied (Figs 1, 2). Instead, however, our study revealed two groups of black yeasts, one in which strains are killed at high rates and the other in which strains are killed to a lesser degree by human neutrophils. The lower echelon of killing rates corresponds to an observed pattern of clinical virulence, with expected progression of many infections to systemic or disseminated invasion. Thus, our study strongly indicates that multiple virulence factors contribute to the high killing resistance of the black yeasts of group II. The factors involved seem to be absent or not expressed in the group I yeasts. Owing to the close phylogenetic relationship of *Exophiala* species [12], acquisition of entirely novel virulence factors by a subset of species is unlikely. Therefore, one can speculate that apparently avirulent species still occupy ecological niches in which expression of the, as yet uncharacterized, non-melanin virulence factor(s) has not yet been activated. Alternatively, subtle differences in the polymerization, deposition patterns, or quantity of melanin produced in the species studied could result in differing levels of effectiveness in scavenging of free radicals [19] and could thus contribute to the observed differences in killing rates.

Further studies have to be conducted to elucidate the virulence factors that confer pathogenicity upon black yeasts. In this respect, *E. dermatitidis* could serve as an ideal model, especially in the knowledge that protocols for genetic transformation and disruption have recently been developed [20] for this relatively well-studied species.

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