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Review Article on Black Fungi

Dr. Arun Patel, Prakash Singh, Sahil Sen.
Shri Ram Group Of Institution Department Of Pharmacy
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INTRODUCTION

Black yeasts have been known since the end of the 19th century, but they still are among the most difficult fungal groups to identify and therefore the knowledge on this group is still only fragmentary.

The diagnostic confusion in the past is not surprising, since the taxonomy of black yeasts is now known to be much more complicated than was anticipated. With the application of molecular crite- ria a great number of undescribed species is encountered. This number is expected to increase even more when detailed studies in biodiversity are performed. Apparently undescribed taxa from the environment and even from human patients are regularly found, and their number is likely to augment exponentially when less commonly explored sources are sampled. It seems probable that within a few years from now the number of taxa known in black yeasts and their relatives will multiply Revealing further teleomorph:

anamorph relationships will be key issues in the study of these organisms.Molecular phylogeny has enabled the attribution of black yeast species to main groups in the fungal king- dom. One of the most interesting findings made in recent years has been the consistent relation of human patho- genic taxa (black yeasts as well as their filamentous counterparts) to a small, clearly delimited group, the order Chaetothyriales, and the family Herpotrichiellaceae in particular.This family is phylogenetically remote from the remaining bitunicate ascomycetes. It has been sug- gested that the fungi went through a process of rapid diversification, probably after having entered a new sub- stratum. It is tempting to speculate that this substratum is the human body.A reliable taxonomic system that reflects natural rela- tionships has predictive value. It provides a clue towards understanding the ecology of species, as species appea display a surprising ecological consistency. Species can be retrieved from their expected habitat after using enrich- ment techniques. Major evolutionary trends in the black yeasts and their allies not only concern human patho- genicity, but also hyperparasitism and osmophily. Each study on virulence factors should begin with detailed consideration of the phylogeny of the organism.

Tracing the source and route of infection of neurotropic black yeasts

The black yeast Exophiala dermatitidis is known from the environment, but also from systemic mycoses in humans. In Southeast Asia fatal cerebral infections are noted in patients which are otherwise in good health. However, the preponderant clinical picture in Europe is

subclinical colonization of the lungs of patients with cystic fibrosis (CF); the rare systemic cases in this part of the world are mild and occur In immunocompromised patients only. The two clinical pictures are partly caused by members of a single population, as has been determined by random amplifiedpolymorphic

DNA (RAPD).The question is whether E.dermatitidis is contaminant:opportunistic fungus only,as might be concluded from its European occurrence,or whether it should be regarded as a sys- temic pathogen, as

seems apparent from its behaviour in Southeast Asia. To address this question, E. dermatitidis was compared with Pseudallescheria boydii, an environmental species showing neurotropism after temporary coma and aspira- tion of contaminated water. The taxon displays a re- markable degree of variability in ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequences and polymerase chain reaction (PCR)- fingerprint data. Within the species, several nuclear DNA homology groups are known, but identical strains (i.e., with \80% homology) vary by \ 10% in their ITS sequences. P. boydii easily forms a teleomorph in culture and thus it is likely to show abundant meiotic recombination. These data indicate that the taxon inhabits a permissive ecolog- ical niche (namely

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polluted, nitrogen-rich, water), where many genotypes that emerge in the course of evolution are able to survive and can occur next to each other. Due to its high degree of recombination the tree shows poor resolution.

E. dermatitidis is much less variable; no sexuality is known. This may indicate that the species is in an active process of adaptation to a new niche. The species was proven to be oligotrophic and thermophilic. These condi- ons are met, for example, in steambaths, which are hot and moist, and have slightly osmotic wall surfaces. This ecology explains the prevalence of the species in the lungs of CF patients. Bathing facilities in Europe were proven to contain several more Exophiala species, each inhabit- ing slightly different microniches determined by tempera- ture relationships. Apparently, oligotrophism is an ecological mainstay. Neurotropism is also a plesiomorph characteristic in relatives of Exophiala, such as Cladophialophora bantiana and Ramichloridium macken- ziei. Hence, combining the two ecological tendencies, E. dermatitidis is likely to be predisposed to adapt as a neurotropic pathogen. Its molecular structure seems to indicate that this event has happened only recently.

Comparison of phagocytosis, oxidative burst and killing of black yeasts

Phylogenetic analysis of black yeasts and their relatives revealed that all type strains of the genus Exophiala clustered as a monophyletic group together with mem- bers of the Herpotrichiellaceae (order Chaetothyriales), indicating a close relationship

[1]. Therefore, it may be expected that they share virulence factors resulting in comparable pathogenicity. The presence of melanin has been considered as an important virulence factor and it was recently shown that this leads to lower killing rates in E.dermatitidis

when comparing melanized strains with a respective albino mutant in a bioassay using whole hu- man blood [2]. Surprisingly,

melanized species considered virulent were found at aphylogenetically short distance to melanized, but virtually non-virulent species,

e.g. E. spinifera and Phaeococcomyces exophialae [3]. Since the most important defense system of the human organism against fungal infections are professional phagocytes (i.e., macrophages and neutrophils releasing reactive oxidative intermediates [ROI] that have been described to be able to kill yeasts and filamentous fungi), the present study addressed this discrepancy assessing phagocytosis, evoked oxidative burst, and killing by human neu- trophils of black yeast species (n¾ 9) exhibiting differ- ent pathogenic potential. A recently developed method for testing phagocytosis of E. dermatitidis and its al- bino mutants by human neutrophils was applied using flow cytometry in combination with a killing bioassay comprising six independent assays [2].

Whereas phagocytosis and the evoked oxidative burst were increasing nearly synchronously during the test pe- riod, surprisingly, the degree of killing differed signifi- cantly after 5 h of co-incubation in whole blood of healthy human donors. Two groups of fungi could be identified that were found to be killed to a high (range 96·4–80·5%; group 1) or low (range 65·7–50·2%; group 2)degree. Group one comprised (data presented as per cent killed after 5 h incubation in whole blood): Can- dida albicans DSM 11943 (95·3%), Saccharomycescere-×isiae DSM1333(94·6%),Hortaea werneckii CBS $107.67^{N\ T}$ (80·5%), E. castellan Nii CBS $158.58^{N\ T}$ (96·4%), Phaeoannellomyces elegans UTMB 1286^{T} (93·2%), P. exophialae CBS 668.76^{T} (86·6%), and the white mutant strains of Exophialadermatitidis mel^{3-} ATCC 44504 (95·0%).

Group two comprised: E. dermatitidis ATCC 34100 (61·0%), E. dermatitidis CBS 207.35^{T} (65·7%), E. jeanselmei ATCC 34123^{T} (50·2%), E. mesophila CBS 402.95^{T} (63·1%), E. bergeri CBS 526.76^{T} (62·8%), and E.spinifera CBS 107.67^{T} (57·1%).

The killing of the non- pigmented yeasts C. albicans and S. cere×isiae was comparable in degree to that seen with the non-melanized E. dermatitidis strain. The de- posited melanin in the cell wall of black fungi is known to absorb light and heat energy due to numerous free carboxyl groups. This accounts for many of the protec- tive, as well as the photosensitizing, properties of melanin. In the case of plant pathogens, it is well known that melanin increases cell wall rigidity and thus it might render killing more difficult. In the case of

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ascomycetous black yeasts, dihydroxynaphthalene(DHN) melanin is formed by oxidative polymerization of phenolic compounds [6]. It can be speculated that the presence of melanin confers a higher capacity to neutralize oxidants, resulting in survival during the evoked oxidative burst in the phagolysosome of neu- trophils. Thus, for all melanized yeasts analyzed in the present study a comparable survival rate would be ex- pected, especially since the degree of phagocytosis and evoked oxidative burst was comparable in all strains studied. Intracellular location of the yeast cells associ ated with the neutrophils was ensured by microscopevaluation of the phagocytosis process [2].

Despite our working hypothesis that due to their close phylogenetic relationship the same type of melanin should be present in all the black yeasts stud- ied, the degree of killing after 5 h differed significantly between the melanized strains studied. The black yeasts that were killed to a degree comparable to that seen in non-melanized strains (i.e., C. albicans, E. dermatitidis mel$3¼$, S. cere×isiae) are mainly isolated from mild hu- man infections, whereas strains killed to a lesser extent are well-known for their potential to cause severe infec- tions, with the exception of E. mesophila. In the latter species its reduced growth at 37 °C might prevent inva- sion of the human host.

Invasiveness of fungal pathogens has often been linked to defects in cell- mediated immunity, but the re- sults of the present study clearly show that neutrophils of healthy donors killed pathogenic melanized species to a lesser extent than other species. Since neutrophils are still considered to be the most important effector cells, low killing rates of the respective species most probably reflect their high virulence. Therefore, the striking differences in killing rates of melanized species strongly indicate that melaninization of the cell wall alone is insufficient to confer the killing resistance.If all black yeasts tested possess the same type and structure of melanin the difference in killing might be attributable to the expression of an additional virulence factor. Due to the close phylogenetic relationship of Exophiala species , acquisition of novel virulence fac- tors is unlikely. Therefore, one can speculate whether expression of suchplesiomorphic virulence factor de- pends upon ecological stress factors. Another explana- tion is that due to the complex composition of melanin, i.e., monomers usually complexed with proteins and carbohydrates [4], differences in final polymerization could result in different linkage patterns of monomers with different a capacity for scavenging radicals which may contribute to the observed differences. Survival in the phagolysosome might subsequently result in its pen- etration and invasion of the surrounding tissue, since melanized hyphae exert larg turgor-derived forces at their apices than non-melanized cells . Definitive proof of the involvement of melanin in thevirulence of black yeasts awaits further experiments by specifically altering DHN-melaninbiosynthesispathway by, for ex- ample, gene disruption. Due to the establishment of genetic transformation, gene disruption protocols and a gene expression system [9], such experiments could be feasible forE. dermatitidis in the near future.

Molecular identification of dematiaceous environmental versus patient strains An attempt was made to findagentsofh chro- moblastomycosi in the environment,on the assumption that the infectionisinitiated by traumatic inoculation and thus that the aetiological agents are likely to be saprobes. In a phylogenetic tree (Fig.1) derived from sequences of ITS1, ITS2and 5·8SrDN weincluded allknown agents ofchromobla stomycosis ,supplemented withmorphological similr environmental strainsan other potentially pathogenic members of the Her- potrichiellaceae. Approximately 10 groups can be recog- nized. Reference strains of Ramichloridium, Rhinocladiella and Fonsecaea formed distinct groups (I, II and VIII).

A group designated asFonsecaea contained, except for reference strains of F. pedrosoi, a number of clinical isolates from patients with chromoblastomycosis but also some saprobes (VIII). The reference strains of F. pedrosoi and F. compacta comprised a subgroup at some distance

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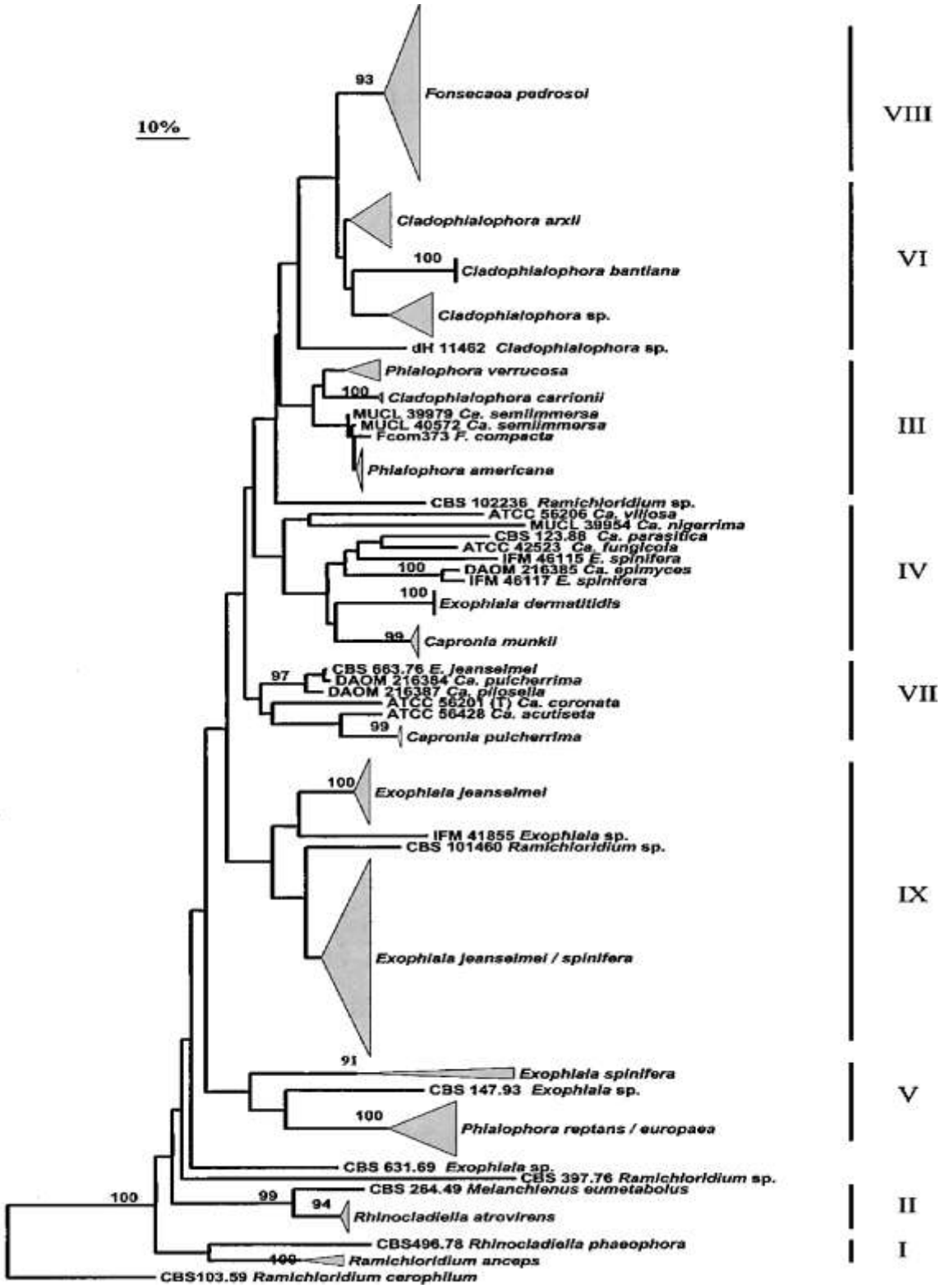

Fig. 1 Phylogenetic tree of agents of chromoblastomycosis and their relatives, based on the ITS1, ITS2 and 5·8S rDNA sequences, using the neighbor joining algorithm.

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C. carrionii in cacti at the chromomycosis semi-arid endemic zone in Venezuela

Chromomycosis is a chronic subcutaneous granuloma- tous disease caused by several melanized dimorphic fungi reported predominantly from tropical countries.

In Venezuela, the first case was described by O'Daly in 1938; in 1943 he also reported for the first time an aetiological agent now known as C. carrionii. The en- demic area is in the Northwest of Venezuela. Since 1959, Borelli noted that patients infected by Fonsecaea pedrosoi came mainly from humid climates, whereas C. carrionii seemed to occur in semi-arid zones. Keeping goats is one of the main agricultural activities in the latter area and over the years labourers repeatedly traumatize themselves with cacti thorns. An increase in persons susceptible to the development

of chromoblastomycosis is thus ob- served.

Chromoblastomycosis is considered to be a multi- factorial disease, involving genetic as well as environmental factors. A prevalence of 16:1000 cases of chromoblastomycosis should be explained by the coinci- dence in the same geographical area of a homogeneous

genetically susceptible population and facility of exposure to the natural source of infection. Since 1983, several studies were carried out at Francisco de Miranda Univer- sity in order to confirm the presence of C. carrionii in the endemic zone [17 – 20].

Samples were collected in the vicinity close to the patients' houses: fragments of cacti, spines, decaying wood and fence bark fragments. Brown erosive lesions in cactus stems were studied. Thin sections of vegetative tissue, spines and wood were carefully examined to search for brown muriform cells. Positive samples were covered with a thin layer of glycerin:yeast, peptone and glucose liquid medium (^{1} volume), placed on a slide upon a bent glass rod into a Petri dish with 5 ml of sterile water to maintain humidity and incubated at room temperature with daily examination. Proteolytic activity and thermo- tolerance tests were carried out to confirm strain identifi-cation.

Several isolates of C. carrionii, one of Sporothrix schenckii, and a number of unidentified fungal species, were repeatedly observed to produce similar spherical, thick-walled cells growing by isotropic enlargement. C. carrionii was detected in 11 localities in association with common xerophytes

Chromoblastomycosis: a therapeuticchallenge

Chromoblastomycosis (CBM) is a chronic, subcutaneous fungal infection, caused by the transcutaneous implanta- tion of several species of dematiaceous fungi. The disease is more frequent in tropical and subtropical regions among rural workers. After traumatic implantation, the initial lesion can evolve into pleomorphic lesions, leading to dense dermal fibrosis and oedema [21,22]. CBM le- sions are recalcitrant and extremely difficult to eradicate. In this manner, patients with CBM are a true therapeutic challenge for clinicians. During the last few decades, several treatment regimens have been employed [23 – 28]. In the early stages, the lesions respond to surgical resec- tion but later, as the severity increases, better results are achieved with chemotherapy. Therapeutic success can be related to the aetiological agent (C. carrionii is more sensitive than F. pedrosoi [29]), to the severity of the disease (oedema and dermal fibrosis can reduce antifun- gal tissue levels) and obviously, to the choice of the antifungal drug [30]. There are no comparative trials in CBM. In most of the clinical trials, the lesions are not graded according to severity and standardized criteria of cure are not used by the different authors dealing with this mycosis. Currently, itraconazole (ITZ) alone or com-bined with flucytosine or topical liquid

nitrogen (cryotherapy) appears to be the best treatment for CBM [28 – 32]. the State of Parana´ , transmission of the disease is mainly occupational, affecting the inhabitants of the State's up- lands. In 48 patients, a non-comparative clinical trial with itraconazole was carried out to evaluate its efficacy and toxicity. Eighteen patients were considered unevalu- able because they failed to return for their control visits or because of non-continuous therapy. The CBM lesions were classified according to morphology and severity. A mild form was defined as a solitary plaque or nodule measuring less than 5 cm in diameter. A moderate form was taken to be solitary or multiple lesions (nodular, verruciform or plaque types), existing alone or in combi- nation, covering one or two adjacent cutaneous regions, and measuring less than 15 cm in diameter. The severe form

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consisted of any type of lesion, alone or in combi- nation, covering extensive cutaneous regions, whether adjacent or non-adjacent [30]. All subjects received itra- conazole at 200 – 400 mg day¼ 1 until the established crite- ria of cure were achieved. Clinical criteria included: disappearance of pain and itching, and complete healing of all lesions with scarring. Mycological criteria were the absence of pathogens on direct microscopic examination and no fungal isolation on culture. Histological criteria included absence of pathogens, atrophy of the epidermis, disappearance of microabscesses and granulomas, re- placement of granulomatous infiltrate by chronic inflam- mation and fibrosis. The persistence of all these findings had to continue for three consecutive monthly biopsies [33]. Clinical, mycological, histopathological and labora- tory evaluations were performed before, during and after therapy. In order to establish whether the chronic itra- conazole therapy could interfere in human steroidogene- si and androgenesis, the adrenal response to cortico- tropin and testosterone was evaluated in15 patients by radioimmunoassay.

This report presents the results obtained with 30 CBM patients treated with itraconazole (Table 1). Nine patients (30%) presented mild CBM lesions with a median of 7·5 (range 1 – 19) years of duration. Four patients (44%) in this group had been treated previously. In 12 patients (40%), the lesions were moderate and had been present for a median time of 20 (range 6 – 50) years. In this group, five patients (42%) referred earlier treatments with anti- fungal drugs. Finally, lesions were typed as severe in nine patients (30%) and were of long duration, median 24 (range 18 – 40) years. Sixteen patients (53%) had been treated previously. Final assessment showed that eight patients (89%) with mild forms achievedclinicaland mycological cure after 10·9 (range 7 – 17·6) months of therapy. No relapses were observed in this group after the mean time of 31·2 (range 12– 72) months. Similar responses were observed in 11 of the 12 patients (91%)

Table 1 Clinical and demographic characteristics of 30 patients wih chromoblastomycosis treated with itraconazole

Clinical form	Clinical and mycological cure n (%)	Duration of treatment (months (median))	Improvement n (%)
Mild	8 (89%)	10·9 (7–17·6)	1 (11%)
Moderate	11 (91%)	12·9 (5–31)	1 (9%)
Severe	4 (44%)	30 (10–51)	5 (56%)
Total	23 (76%)	18	7 (24%)

with moderate forms, after an average of 12·9 (range 5 – 31) months of continuous treatment. In this group, one patient relapsed after 6·3 months of follow-up while the remaining patients did not relapse (12 – 60 months follow-up). Among the nine patients with severe CBM lesions, four (44%) had clinical and mycological response after a mean of 30 (range 10 – 51) months of treatment, and the remaining patients had improved significantly. One relapse was observed during the follow-up (after 35 months), but the patient improved again after a new course of therapy. No significant changes in the values of hematological and biochemical tests were observed.

Mean cortisol and testosterone concentrations at base- line were 12·4 mg dl¼ 1 and 454 ng dl¼ 1, respectively, and after 12·495·2 months of treatment with itraconazole were 15·4 mg dl¼ 1 and 480 ng dl¼ 1, respectively. There was no clinical or laboratory evidence of

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steroidogenic or androgenic impairment [34].

These results show that the therapy with itraconazole can achieve long lasting clinical and mycological cures in most of the patients having mild to moderate forms of CBM, after prolonged periods of treatment. On the other hand, only 44% of the severe cases were cured clinically and mycologically. The clinical outcome observed in those patients presenting severe lesions of CBM, could be related todecreased itraconazole tissue concentrations. Local fibrosis, oedema and bacterial co- infectionare common associated factors that can decrease local itra- conazole concentration, especially in the subcutaneous tissues, which in severe lesions are replaced by dense fibrosis.

Other therapeutic strategies available include the com- bination of itraconazole with flucytosine and:or the asso- ciation of local liquid nitrogen [31,32]. Both methods may reduce the duration of itraconazole treatment. Ac- cording to preliminary data, terbinafine at a daily dose of 500 mg for 6 – 12 months also seems to be effective in CBM (efficacy 85%). However, the results presented by Esterre et al. [35]

Contributors

The contributors to this symposium were: **G. S. de Hoog,**

D. **Attili Angelis, A. H. G. Gerrits van den Ende, T. Matos, A. A. Pizzirani- Kleiner, J. Rainer & V. Vicente,** Tracing the source and route of infection of neurotropic black yeasts ; **G. Haase & H. Peltroche- Llacsahuanga,** Comparison of phagocytosis, oxidat i×e burst and killing of black yeasts with different pathogenic potential ; **V. Vi- cente,** Molecular identißcation of dematiaceous en×iron- mental ×s. patient strains ; **G. Zeppenfeldt-**

cannot be compared with our results because different assessment criteria were employed in both trials [30,35]. In the future, the new antifungal drugs under develop- ment may play an important role in the treatment of CBM. In ×itro dematiaceous fungi are very sensitive to **Fernandez, N. Richard- Yegres & F. Yegres,** Cladophialophora carrionii in cacti at the chromomycosis semi-arid endemic zone in Venezuela ; **F. Queiroz-Telles,** Chromoblastomycosis: a therapeutic challenge. The co- convenors were **G. S. de Hoog** and **F. Queiroz-Telles.**

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