Virulence and Resistance to Antifungal Therapies of *Scopulariopsis* Species

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Scopulariopsis is an emerging opportunistic fungus characterized by its high resistance to antifungal therapies. We have developed a murine model of disseminated infection in immunosuppressed animals by intravenous inoculation of *Scopulariopsis brevicaulis* and *Scopulariopsis brumptii*, the most clinically relevant species, in order to evaluate their virulence and their responses to conventional antifungal treatments. Survival and tissue burden studies showed that *S. brumptii* was more virulent than *S. brevicaulis*. The three drugs tested, liposomal amphotericin B, posaconazole, and voriconazole, prolonged the survival of mice infected with *S. brumptii*, but none showed efficacy against *S. brevicaulis*. The different therapies were only able to modestly reduce the fungal burden of infected tissue; however, in general, despite the high serum levels reached, they showed poor efficacy in the treatment of the infection. Unfortunately, the most effective therapy for *Scopulariopsis* infections remains unresolved.

The fungal genus *Scopulariopsis* of the ascomycetes includes hyaline and melanized species that are soil saprobic and show a wide geographical distribution. *Scopulariopsis* species are commonly isolated from air, wood, decaying organic matter, manure, and animal remains (1), but they are occasionally involved in human infections. They are mainly related to onychomycosis (2, 3), keratitis (4), otomycosis (5), and cutaneous infections (6), although disseminated infections have also been linked to high mortality rates in immunosuppressed (7, 8, 9) and more rarely in immunocompetent patients (10, 11). The most common species involved in human infections are *Scopulariopsis brevicaulis*, *Scopulariopsis gracilis*, *Scopulariopsis brumptii* (currently renamed as *Microascus paisii* [12]), *Scopulariopsis candida*, and their relatives *Microascus cirrosus* and *Microascus cinereus* (7, 13). There are very few clinical and experimental data available on the management of infections by *Scopulariopsis*. *In vitro* susceptibility studies have shown high rates of resistance of these fungi to practically all current antifungal agents (14, 15, 16), which makes it difficult to treat such infections successfully. Surgery combined with antifungal therapy has been recommended for the treatment of infections by *Scopulariopsis* species, although no particular antifungal agent is mentioned (17). The aim of the present study was to develop murine models of invasive infections by two clinically relevant species in order to evaluate the efficacy of liposomal amphotericin B (LAMB), voriconazole (VRC), and posaconazole (PSC), since they are the most commonly used drugs against infections by filamentous fungi (17, 18).

MATERIALS AND METHODS

Fungal isolates and inoculum preparation. Two strains of *S. brevicaulis* (FMR 12216 from synovial fluid and FMR 12246 from aorta tissue) and two strains of *S. brumptii* (FMR 12240 from bronchoalveolar lavage fluid and FMR 12229 from sputum) were included in the study. The isolates were identified by sequencing the D1/D2 domains of the 28S rRNA gene and a fragment of the elongation factor 1- α gene (*EF1- α*) (13) and by comparing the sequences with those of the type strains. The *in vitro* antifungal susceptibility tests were carried out following CLSI guidelines (19). Fungi were grown on potato-carrot agar (PCA; 20 g of filtered potatoes and carrots plus 20 g of agar in 1 liter of distilled water) for 5 days at 25°C. On the day of infection, cultures were flooded with sterile saline and were

filtered through sterile gauze to remove clumps of conidia and hyphae. The resulting suspensions were adjusted by haemocytometer counts, and to confirm viability, 10-fold dilutions were cultured on PCA.

Animals. Male OF1 mice (Charles River; Criffa SA, Barcelona), with a mean weight of 30 g, were used in the assays. Mice were housed in standard conditions with *ad libitum* access to food and water. Mice were rendered neutropenic by an intraperitoneal (i.p.) injection of 200 mg/kg of body weight of cyclophosphamide (Genoxal; Laboratorios Funk SA, Barcelona, Spain) plus 150 mg/kg of 5-fluorouracil (Fluorouracil; Ferrer Farma SA, Barcelona, Spain) given intravenously (i.v.) 1 day prior to the infection. This immunosuppression has demonstrated peripheral blood polymorphonuclear leukocyte (PMN) counts of <100 PMNs/ml (20). All animal procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

Virulence study. The virulence of *Scopulariopsis* spp. was evaluated against immunocompetent and immunosuppressed mice.

The strain *S. brevicaulis* FMR 12246 was selected randomly to evaluate virulence in immunocompetent mice. Two groups of 8 mice were challenged with 1×10^5 or 1×10^7 CFU/animal, respectively. Inoculum was administered i.v. in 200 μ l of sterile saline via the lateral tail vein.

In the immunosuppressed mice model, animals were rendered neutropenic 1 day prior to infection as explained above. We tested the two strains of *S. brevicaulis* and the two strains of *S. brumptii* mentioned previously by challenge with 1×10^5 , 1×10^6 , or 1×10^7 CFU/animal. Additionally, the strain FMR 12246 of *S. brevicaulis* was tested with 5×10^5 CFU/animal. Experimental groups consisted of 16 mice per inoculum (8 for survival studies and 8 for fungal load and histopathology). Animals were checked twice daily for 15 days postinfection. In order to compare results, mice included in the tissue burden study were euthanized on day 6 postinfection when controls began to die.

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Tissue burden and histopathology studies. After euthanasia, the kidneys, lungs, spleens, livers, and brains of mice were aseptically removed, and approximately half of each organ was weighed and mechanically homogenized in 2 ml of sterile saline. Serial 10-fold dilutions of the homogenates were placed onto PCA and were incubated for 3 days at 25°C for CFU per gram calculation. For the histopathology study, the remaining portion of each organ was fixed with 10% buffered formalin, dehydrated, paraffin embedded, and sliced into 2- μ m sections, which were stained with hematoxylin-eosin (H&E), periodic acid-Schiff (PAS) stain, and Grocott methenamine silver (GMS) for examination by light microscopy.

Treatments. Due to the low mortality rate observed in immunocompetent mice, the efficacy of the antifungal treatments was evaluated only in immunosuppressed animals inoculated with 5×10^5 CFU of *S. brevicaulis* FMR 12246 and 1×10^6 CFU of *S. brumptii* FMR 12240, which produced acute infections with all mice dying within 15 days. The treatments evaluated were LAMB (Gilead Sciences S.A., Madrid, Spain) given at 10 mg/kg i.v. once a day (QD), PSC (Noxafil; Schering-Plough Ltd., Hertfordshire, United Kingdom) at 20 mg/kg given orally (p.o.) by gavage twice daily (BD), or VRC (Vfend; Pfizer S.A., Madrid, Spain) at 60 mg/kg p.o. by gavage QD. These doses allowed serum drug concentrations to be higher than the respective MICs (21, 22). From 3 days before infection, mice receiving VRC were given grapefruit juice instead of water (23). All treatments began 1 day after challenge and lasted for 5 days. To prevent bacterial infections, mice received 5 mg/kg/day of ceftazidime (Ceftazidima; Normon, Madrid, Spain) subcutaneously. Animals from the survival study were checked twice a day for 15 days after challenge, while animals from the fungal load study were euthanized at day 6 postinfection, 4 h after the last dosing.

Bioassay. Mice from the fungal burden group were anesthetized by inhalation of sevoflurane (Sevorane; AbbVie, Madrid, Spain), and 1 ml of blood was obtained by cardiac puncture. Blood was centrifuged, and the obtained serum was used to determine concentrations of LAMB, PSC, and VRC by diffusion assay following previous studies (24, 25). In brief, 22 ml of yeast nitrogen base (YNB; 6.9 g/liter nitrogen yeast base, 10 g/liter peptone dextrose, 5 g dextrose, and 15 g/liter of agar) and an inoculum of *Candida parapsilosis* ATCC 22019 at 2×10^6 CFU/ml were mixed at 45°C to 50°C and were poured into a sterile petri dish. The plates were allowed to solidify at room temperature. Then, 4-mm-diameter wells were perforated with a sterile borer. Thirty microliters of each standard of LAMB, PSC, VRC, and serum samples were dispensed in the wells. Plates were incubated at 35°C for 24 h. The diameter of growth inhibition was measured and was used to determine drug concentration in samples by linear regression analysis. Tests were carried out in duplicate.

Statistical analysis. The mean survival times (MST) were estimated by the Kaplan-Meier method and were compared among groups by using the log rank test. In tissue burden studies, colony counts were $\log_{10}$ transformed and were compared by the two-tailed Mann-Whitney U test, using GraphPad Prism 6 for Windows. *P* values of ≤ 0.05 were considered statistically significant.

RESULTS

Virulence study. *S. brevicaulis* FMR 12246 showed a reduced virulence in immunocompetent animals with 100% and 80% survival after infection with 1×10^5 and 1×10^7 CFU/animal, respectively (data not shown). Due to this low mortality rate and as mentioned above, only immunosuppressed animals were used in subsequent experiments.

In immunosuppressed mice, the lowest inoculum tested, i.e., 1×10^5 CFU/animal, caused the deaths of 62.5% of animals infected with either of the two strains of *S. brevicaulis* and caused 87.5% and 100% mortality in mice infected with strains FMR 12240 and FMR 12229 of *S. brumptii*, respectively, while the highest inoculum caused the death of all of the animals within 15 days after challenge (Fig. 1).

Table 1 shows the results of the tissue burden studies. Mice infected with any strain showed detectable fungal load on day 6 postinfection in the five organs tested. Quantitative cultures correlated with the size of the inoculum tested, and the animals infected with the highest inoculum showed the highest value of CFU per gram. The most affected organs after infection by *S. brevicaulis* were lung and spleen, while *S. brumptii* majorly affected the spleen and liver. Histological findings at day 6 postinfection showed a clear fungal invasion with the presence of hyphae in all of the organs. Lungs were particularly affected, which displayed interstitial disease, with vascular congestion, focal atelectasis, and alveolar hemorrhage. Spleens showed congestion in the sinuses. Dilated and congested vessels were observed in liver and kidney tissue. Neither inflammatory response nor necrosis was observed. No differences were found between species.

Drug efficacy study. The MICs of amphotericin B (AMB), VRC, and PSC against *S. brevicaulis* FMR 12216 and *S. brumptii* FMR 12229 were $>16 \mu\text{g/ml}$ for all three compounds, while those against *S. brevicaulis* FMR 12246 were $>16 \mu\text{g/ml}$, $4 \mu\text{g/ml}$, and $2 \mu\text{g/ml}$ and those against *S. brumptii* FMR 12240 were $>16 \mu\text{g/ml}$, $4 \mu\text{g/ml}$, and $1 \mu\text{g/ml}$, respectively.

The three drugs significantly prolonged the survival of mice infected with *S. brumptii* compared with that of the control group ($P \leq 0.0037$), while none of them showed efficacy against *S. brevicaulis* ($P \geq 0.13$) (Fig. 2).

Although the three drugs tested moderately reduced the fungal load of some of the organs studied, none of them was able to reduce the tissue burden in all of the organs tested in any of the two strains evaluated. (Fig. 3).

After the completion of the treatment, PSC and VRC levels in serum (mean $\pm$ standard deviation, 5.02 ± 0.77 and $12.42 \pm 0.97 \mu\text{g/ml}$, respectively) were above the corresponding MICs ($4 \mu\text{g/ml}$ and $\leq 2 \mu\text{g/ml}$, respectively). Despite the high dose of LAMB given, the serum concentration of this drug ($15.41 \pm 1.56 \mu\text{g/ml}$) was lower than the AMB MIC value ($>16 \mu\text{g/ml}$).

DISCUSSION

Although aspergillosis is the most frequent invasive mold infection, severe infections by other opportunistic filamentous fungi are emerging, especially in immunocompromised populations (26, 27). *Scopulariopsis*, although a rare fungus, infects humans too and, contrary to aspergillosis, there are no recommended therapies. *Scopulariopsis* is mainly associated with localized infections in immunocompetent patients (2, 3). More rarely, invasive infections by this fungus have also been described in neutropenic patients. The delayed diagnosis and the high level of resistance of *Scopulariopsis* to conventional antifungals (13, 14, 16) are responsible for the high mortality associated with disseminated infections (5).

To our knowledge, this is the first animal study that has explored the virulence of *Scopulariopsis*. In general, immunocompetent mice showed a high survival rate despite the high inocula employed. This is probably due to the efficacy of the immune system in controlling the infection, which explains the low number of reported cases of invasive infections in immunocompetent patients (11). In contrast, our results show high virulence of the four strains tested in immunosuppressed animals because all of the animals died after being challenged with 5×10^5 , 1×10^6 , and 1×10^7 CFU, and high fungal loads were recovered from all studied organs. Animals infected with *S. brumptii* at 1×10^5 CFU also

FIG 1 Cumulative mortality of immunosuppressed mice (8 mice per experimental group) infected with *S. brevicaulis* FMR 12246 (A) and FMR 12216 (B) and *S. brumptii* FMR 12240 (C) and FMR 12229 (D). ^a, *P* value of <0.05 versus 1×10^5 CFU/animal; ^b, *P* of <0.05 versus 5×10^5 CFU/animal; ^c, *P* of <0.05 versus 1×10^6 CFU/animal.

showed a lower survival rate than those challenged with *S. brevicaulis*, which suggests that it is more virulent. In terms of fungal load, the two species similarly invaded all of the studied organs; the lungs and liver were the most affected after infection by *S.*

brevicaulis, and the liver and spleen were the most affected in cases of *S. brumptii* (Table 1).

Histopathological and tissue burden findings were similar among species, and all of the strains tested were able to disseminate.

TABLE 1 Fungal load of different organs on day 6 postinfection from immunosuppressed mice infected with the indicated inoculum of *Scopulariopsis* spp.^a

Species and strain	Inoculum, CFU/animal	Mean log ₁₀ CFU/g of tissue ± SD				
		Liver	Kidney	Brain	Spleen	Lung
<i>S. brevicaulis</i> FMR 12246	1×10^5	3.34 ± 0.05	1.92 ± 0.09	0.71 ± 0.53	2.54 ± 0.25	3.41 ± 0.06
	5×10^5	3.29 ± 0.08	3.12 ± 0.11	2.56 ± 0.3	3.43 ± 0.25	4.66 ± 0.2
	1×10^6	3.85 ± 0.08	3.78 ± 0.15	2.88 ± 0.08	4.19 ± 0.16	5.19 ± 0.19
	1×10^7	5.73 ± 0.04	3.64 ± 0.18	3.32 ± 0.14	5.75 ± 0.08	5.42 ± 0.25
<i>S. brevicaulis</i> FMR 12216	1×10^5	3.8 ± 0.24	1.72 ± 0.11	0.63 ± 0.5	4.68 ± 0.2	1.29 ± 0.63
	1×10^6	4.46 ± 0.24	2.61 ± 0.27	1.41 ± 0.24	4.52 ± 0.25	3.32 ± 0.54
	1×10^7	5.64 ± 0.16	3.55 ± 0.11	2.3 ± 0.16	5.8 ± 0.09	3.89 ± 0.05
<i>S. brumptii</i> FMR 12240	1×10^5	3.9 ± 0.03	3.02 ± 0.18	0.89 ± 0.58	4.15 ± 0.11	2.14 ± 0.05
	1×10^6	5.04 ± 0.19	3.98 ± 0.06	2.11 ± 0.07	5.31 ± 0.18	3.77 ± 0.48
	1×10^7	5.86 ± 0.33	5.05 ± 0.3	2.83 ± 0.03	6.05 ± 0.42	4.87 ± 0.27
<i>S. brumptii</i> FMR 12229	1×10^5	3.9 ± 0.03	0.73 ± 0.81	0.28 ± 0.44	3.73 ± 0.04	1.85 ± 0.11
	1×10^6	5.12 ± 0.12	3.24 ± 0.29	1.43 ± 0.18	5.2 ± 0.19	3.18 ± 0.24
	1×10^7	6.11 ± 0.11	4.69 ± 0.23	2.57 ± 0.09	6.32 ± 0.1	4.36 ± 0.14

^a Data correspond to 8 animals per experimental group.

FIG 2 Cumulative mortality of immunosuppressed mice (8 mice per experimental group) infected with *S. brevicaulis* FMR 12246 (A) or *S. brumptii* FMR 12240 (B) treated with liposomal amphotericin B at 10 mg/kg QD (LAMB 10), posaconazole at 20 mg/kg BID (PSC 20 BID), or voriconazole at 60 mg/kg QD (VRC 60). ^a, *P* value of ≤ 0.05 versus control.

nate to all studied organs, including the brain. These findings agree with clinical reports where disseminated infection by *Scopulariopsis* has been reported to involve multiple organs, including the liver, spleen, kidney, and brain (5). However, conidia, swollen structures, or ascospores were not found as described in some reports (7), which is possibly due to the short period of infection.

Guidelines for hyalohyphomycosis do not recommend any

particular antifungal treatment for invasive infections (17). However, invasive *Scopulariopsis* infections are challenging to treat and are often fatal despite aggressive medical and surgical management. Some response to LAMB (28) and VRC (29) has been occasionally documented, while PSC has only shown good *in vitro* activity. Nevertheless, no therapy has been experimentally tested against this infection previously. In the absence of relevant clinical

FIG 3 Effects of the antifungal treatments on colony counts of organs in immunosuppressed mice (8 animals per experimental group) infected with *S. brevicaulis* FMR 12246 (A) or *S. brumptii* 12240 (B) 6 days after infection. LAMB 10, liposomal amphotericin B at 10 mg/kg QD; PSC 20 BID, posaconazole at 20 mg/kg BID; VRC 60, voriconazole at 60 mg/kg QD. ^a, *P* value of ≤ 0.05 versus control; ^b, *P* value of ≤ 0.05 versus LAMB 10; ^c, *P* value of ≤ 0.05 versus PSC 40; ^d, *P* value of ≤ 0.05 versus VRC 60.

data and of standard therapies linked to a favorable outcome for patients, animal models can play an important role in guiding the use of empirical treatments (18). In the present study, *S. brevicaulis* and *S. brumptii* showed high MIC values against AMB, although LAMB was effective in prolonging the survival of mice infected with *S. brumptii*.

Only PSC and VRC concentrations in serum were above the respective MICs, which agrees with previous studies (30, 31, 32). Despite that, they were only effective against *S. brumptii*. The fungal burdens of the two strains tested were reduced only slightly by the three antifungals.

In summary, the lack of correlation between *in vitro* and *in vivo* studies and the poor efficacy of antifungals make it difficult to manage this infection, which underlines the need for further studies to explore therapeutic alternatives. Synergistic *in vitro* interactions of antifungals against *S. brevicaulis* have been reported (15) and might be a new line of research for the treatment of *Scopulariopsis* infections.

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