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Sterol Compositions and Susceptibilities to Amphotericin B of Environmental *Cryptococcus neoformans* Isolates Are Changed by Murine Passage

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Previous studies have shown that sequential isolates from patients with persistent *Cryptococcus neoformans* meningoencephalitis can vary in sterol composition and susceptibility to antifungal drugs. To investigate the potential of host factors as mediators of this phenomenon, we compared fungal susceptibilities of environmental and clinical isolates from a limited geographic area. Clinical isolates were less susceptible to amphotericin B than environmental isolates. Five environmental isolates were passaged through BALB/c murine hosts; the passaged isolates had changes in sterol composition and reduced amphotericin B susceptibilities relative to those of the parent isolates. In contrast, murine passage of these isolates did not alter their susceptibilities to fluconazole. The results confirm that changes in sterol composition and antifungal susceptibility can occur in vivo as a result of host factors and suggest that human infection can result in selection of variants with reduced susceptibilities to amphotericin B.

Cryptococcus neoformans is an opportunistic fungal pathogen which causes a life-threatening meningoencephalitis in 6 to 8% of patients with AIDS (3, 10). The infection is usually incurable in AIDS patients, and survivors of the initial infection are treated with lifelong antifungal suppressive therapy (18). Amphotericin B remains the mainstay of therapy for initial treatment of acute infection, and fluconazole therapy is routinely used for chronic suppression to prevent relapses (13). Amphotericin B is a polyene antibiotic which has a complex mechanism of action and is believed to mediate fungicidal effects by binding to membrane sterols with subsequent damage to fungal cell membranes. Amphotericin B is also a potent immunomodulator which may contribute to its in vivo efficacy (17). Fluconazole is a triazole compound which is believed to interfere with fungal sterol synthesis (6). For *C. neoformans*, fluconazole may function by inhibiting both 14α-demethylase-dependent and -independent pathways of sterol synthesis (8). Although resistance to antifungal drugs has not been a major problem with *C. neoformans*, some well-documented cases of resistant strains have been reported (5, 12).

Recently we reported on the characteristics of the sterol composition and antifungal susceptibility of initial and relapse *C. neoformans* isolates from five patients with recurrent cryptococcal meningoencephalitis (8). Strain typing with discriminatory DNA probes revealed that initial and relapse isolates from each patient had the same restriction fragment length polymorphism (RFLP) patterns, strongly suggesting persistence of the same strains (2, 14). All five patients received fluconazole therapy, and three had also received amphotericin B treatment in the interval between initial diagnosis and relapse. Relapse isolates differed from the initial isolates in sterol composition, and some relapse isolates also differed in susceptibility to fluconazole. No relapse isolates exhibited a change in amphotericin B susceptibility. These results suggested in vivo selection of *C. neoformans* variants with altered sterol compositions and antifungal susceptibilities. We previously hypothesized that these changes could be the result of selective pressures exerted by the antifungal drug regimen received or possibly by host factors exerted during infection (8). Theoretically, in vivo selection pressures during mammalian infection could result in altered sterol composition or sterol metabolism which could then be reflected in corresponding changes in antifungal susceptibility. Previous investigations of the molecular epidemiology of environmental and clinical *C. neoformans* isolates (4) provided a unique opportunity to compare antifungal susceptibilities of these environmental and clinical isolates. Observed differences could then be experimentally evaluated by passaging environmental isolates in murine hosts with subsequent characterization of sterol composition and antifungal susceptibility.

This report evaluates amphotericin B and fluconazole sensitivities of 7 environmental (pigeon excreta) and 14 clinical isolates obtained from a limited geographic area in Bronx, N.Y., during 1992. Observed differences between clinical and environmental isolates were experimentally evaluated by passaging five environmental *C. neoformans* strains in murine hosts. Subsequently, pre- and postpassage isolates were characterized for their sterol compositions and antifungal susceptibilities.

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MATERIALS AND METHODS

***C. neoformans* isolates.** Fourteen clinical isolates were obtained from the Bronx Municipal Hospital Center (Jacobi Hospital), Bronx, N.Y., and have been described elsewhere (4). All isolates were obtained from human immunodefi-

TABLE 1. MIC_{50}s of amphotericin B and fluconazole for clinical and environmental isolates of *C. neoformans*

Strain	MIC_{50} (μg/ml) of:	
	Amphotericin B	Fluconazole
Clinical		
J10	0.125	2.0
J11	0.25	1.0
J12	0.25	1.0
J13	0.5	1.0
J15	0.25	4.0
J16	1.0	2.0
J17	0.125	16.0
J19	0.5	1.0
J20	0.125	1.0
J22	1.0	1.0
J23	0.5	2.0
J24	0.0625	4.0
J25	0.125	16.0
J26	0.0625	0.25
Environmental		
M1	0.125	2.0
B3	0.0625	8.0
B4	0.0625	1.0
B5	0.0625	0.5
B7	0.0625	0.5
B10	0.0625	0.25
B17	0.0625	8.0

ciency virus-infected patients with their initial diagnosis of cryptococcal meningitis. No patient had received prior systemic antifungal treatment or prophylaxis. The seven environmental isolates used in this study have also been described elsewhere (4) and were obtained from sites contaminated with pigeon excreta in Bronx, N.Y.

All isolates were obtained during the same calendar year. Both clinical and environmental isolates were purified by streaking to a single colony twice and identified by standard microbiological techniques (4). All isolates were determined to be *C. neoformans* var. *neoformans* with canavanine-glycine-bromothymol blue agar (4).

Five of the seven environmental isolates were subsequently passaged through murine hosts (M1, B3, B4, B5, and B7). CNRE-1 RFLP analysis indicated that four of the five isolates were genetically distinct. Isolates B3 and B7 had identical CNRE-1 RFLP patterns.

Murine passage. *C. neoformans* was grown in Sabouraud dextrose broth (Difco Laboratories, Detroit, Mich.), washed two times with 0.02 M phosphate-buffered saline, pH 7.2 (PBS), and suspended in PBS. BALB/c mice were anesthetized with methoxyflurane and given intracerebral infections by injecting 10^5 yeast cells directly into the midline of the cranium at a point 2 to 3 mm posterior to the eyes (11). Intracerebral infection resulted in a meningitis and hydrocephalus in all injected mice. All of the passaged isolates were recovered when the mice died. The passage times for isolates M1, B3, B4, B5, and B7 were 61, 87, 63, 30, and 61 days, respectively. Recovered isolates were then streaked to a single colony and used for further analysis.

Sterol extraction and analysis. *C. neoformans* strains were grown as a shake culture (200 rpm) in yeast nitrogen base broth with amino acids (Difco Laboratories) supplemented with 0.5% (wt/vol) glucose (YNB) at 37°C for 48 h. Total sterols from all strains were extracted at the early stationary growth phase as described previously (8). The sterols were fractionated by thin-layer chromatography using Silica Gel G plates (0.25 mm) and 40 to 60°C petroleum ether-diethyl ether (3:1, vol/vol) as a developing solvent mixture. Sterols were detected by spraying the thin-layer chromatography plate with 50% (vol/vol) sulfuric acid then charring it at 220°C. The sterol fractions were identified by comparison of their R_f values with commercially available standards.

The extracted sterols were quantified by gas-liquid chromatography (GLC). Sterols were derivatized to their trimethylsilyl form according to the method of Vandenheuvel and Court (16). Briefly, sterols (2 mg) were reacted in a glass-stoppered flask with 100 μl of hexamethyl disalazane and 100 μl of 10% (vol/vol) trimethyl chlorosilane in chloroform. The reagents were added in that order and mixed briefly. The reaction mixture was left at room temperature for at least 4 h in the dark. Next, the excess solvent and reagents were removed by evaporation under N_2 gas. Fifty microliters of hexane was then added to the flask to dissolve the reaction mixture. One-microliter portions were analyzed by GLC using an OV-1 column (3% on 100/120 gaschrome Q) in a Vista 6000 (Varian, Sugarland, Tex.) gas chromatograph. The samples were eluted at 230°C with helium as a carrier gas (30 ml/min), the injection temperature was 250°C, and the nonsaponifiable lipids were detected with a flame ionization detector at 300°C. Individual components were identified by comparison of the retention times relative to ergosterol and with those of commercially available standards. GLC peaks were quantified with a Varian Vista 402 integrator. To minimize quantitative errors, cholesterol was included in the GLC analysis as an internal standard. Comparison of retention times of the peaks with those of reference standards by using polydimethylisiloxane as a second column was also performed. Unless otherwise specified, all chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Susceptibility testing. MICs of amphotericin B and fluconazole for *C. neoformans* were determined by the microdilution technique (7). Briefly, YNB buffered to pH 7.0 with 0.05 M morpholinepropanesulfonic acid (MOPS) was used. Antifungal agents were diluted in YNB to provide a stock solution. Drug-free YNB was dispensed into all wells of microtiter assay plates. The twofold dilutions each of amphotericin B and fluconazole stock solution were made by addition to wells numbered 2 through 11 (concentration ranges were 64 to 0.0625 μg/ml). Well 1 served as a sterility control and blank for the spectrophotometric assays; well 12 served as a growth control. The wells were inoculated with 10 μl of yeast cells to achieve a final inoculum of approximately 10^4 CFU/ml. Plates were incubated for 48 h at 35°C without shaking. Yeast cell growth was determined by measurements of the optical density with an automatic microplate reader (Dupont Instruments, Wilmington, Del.) after agitation of the plates for 15 s with a vortex shaker (Vortex-Genie 2 mixer; Scientific Industries, Inc., Bohemia, N.Y.). The MIC endpoint (MIC_{50}) was defined as the lowest drug concentration exhibiting 50% (or more) reduction of optical density at 420 nm compared with that in the control well (7).

Statistical analysis. Comparison of amphotericin B and fluconazole MIC_{50} data for the 7 environmental and 14 clinical isolates was done by *t* test. Comparison of amphotericin B and fluconazole MIC_{50} data for pre- and postpassage isolates was done by paired *t* test analysis. Statistical analyses were performed using the Primer of Biostatistics: The Program Computer Package (McGraw-Hill Publishing, New York, N.Y.).

RESULTS

Sensitivity data of environmental and clinical isolates. Amphotericin B and fluconazole MIC_{50}s for each environmental and clinical *C. neoformans* isolate are summarized in Table 1. Amphotericin B MIC_{50}s ranged between 0.0625 and 0.125 μg/ml for the 7 environmental isolates and between 0.0625 and 1.0 μg/ml for the 14 clinical isolates. The mean amphotericin B MIC_{50} for clinical isolates was significantly higher than the mean MIC_{50} for environmental isolates by *t* test analysis ($P = 0.03$). Fluconazole MIC_{50}s ranged between 0.25 and 8.0 μg/ml for environmental isolates and between 0.25 and 16 μg/ml for clinical isolates. Mean fluconazole MIC_{50}s for environmental and clinical isolates were not statistically significant by *t* test analysis ($P = 0.71$).

Sensitivity data of pre- and postpassage environmental isolates. Amphotericin B and fluconazole MIC_{50}s for the five pre-

TABLE 2. MIC_{50}s of fluconazole and amphotericin B for pre- and postpassage environmental cryptococcal isolates

Drug	MIC_{50} (μg/ml) for[a]:									
	M1		B3		B4		B5		B7	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Amphotericin B	0.12	1	0.062	1	0.062	0.5	0.062	1	0.062	0.25
Fluconazole	2	16	8	16	1	4	0.5	2	0.5	1

[a] Pre and Post, pre- and post-murine passage.

TABLE 3. Total sterol contents of environmental isolates pre- and post-murine passage

Strain[a]	Extracted sterols (mg)	Wt of wet cells (g)	Ratio of sterols to wet wt (%)
M1	71.4	2.93	2.4
M1M	54.6	1.9	2.9
B3	121	2.94	4.1
B3M	100.3	2.73	3.7
B4	75	3.65	1.9
B4M	54.8	4.06	1.3
B5	133.6	6.03	2.2
B5M	86.3	5.1	1.7
B7	49.3	3.73	1.3
B7M	29.2	1.85	1.6

[a] Suffix M denotes murine-passaged isolates.

and postpassage environmental isolates are summarized in Table 2. Amphotericin B MIC_{50}s for the prepassage environmental isolates ranged from 0.0625 to 0.125 μg/ml and for the postpassage isolates ranged from 0.25 to 1.0 μg/ml. In all cases, the amphotericin B MIC_{50}s of passaged isolates were increased 4 to 16 times relative to prepassage MIC_{50}s. Amphotericin B MIC_{50}s for passaged isolates were significantly higher than MIC_{50}s for prepassage isolates by paired comparison *t* test ($P = 0.01$). Fluconazole MIC_{50}s for the prepassage environmental isolates ranged from 0.5 to 8.0 μg/ml and for postpassage isolates ranged from 1.0 to 16 μg/ml. In all cases, the fluconazole MIC_{50}s for passaged isolates were increased relative to prepassage MIC_{50}s, but paired comparison *t* test analysis did not detect statistically significant differences between pre- and postpassage fluconazole MIC_{50}s ($P = 0.10$).

Sterol contents of pre- and postpassage environmental isolates. The total sterol contents of environmental *C. neoformans* isolates before and after passage are summarized in Table 3. Prior to passage the total sterol content by weight ranged between 1.3 and 4.1%, and after passage it ranged between 1.4 and 3.7%. The total sterol content decreased slightly for three isolates after passage and increased for two isolates. There was considerable variation in the sterol compositions of environmental isolates prior to passage. The predominant sterols were obtusifoliol and ergosterol (Table 4), which together composed 70 to 90% of the total sterol content. In four of five environmental isolates, obtusifoliol was the predominant sterol, and ergosterol was the predominant sterol in one. Squalene, calciferol, zymosterol, lanosterol, and 24-methylenedihydrolanosterol were detected in the majority of isolates and individually composed <11% of the total sterol content (Table 4).

There were alterations in the sterol compositions of all five isolates with murine passage. In four of five isolates (M1, B3, B4, and B7) the ergosterol content increased after murine passage and the obtusifoliol content was reduced, suggesting reciprocal changes in the two predominant sterols. No detectable changes in squalene, calciferol, zymosterol, lanosterol, and 24-methylenedihydrolanosterol composition related to murine passage were noted.

DISCUSSION

Despite the frequency of cryptococcal infections in patients with AIDS and the widespread use of drugs that affect sterol metabolism for therapy, relatively little is known about the sterol composition of *C. neoformans*. Kim et al. (9) studied two strains and reported that the principal sterol was epifungisterol. Vanden Bossche et al. studied one strain and reported that ergosterol was the major sterol (15). Recently we analyzed 13 clinical isolates and found that the predominant sterols were obtusifoliol (9 strains) and ergosterol (3 strains) (8). All strains studied to date in relation to their sterols have been clinical isolates. Analysis of five environmental strains in this study revealed that obtusifoliol and ergosterol were the predominant sterols, composing 70 to 90% of the total sterols present. For four of five strains the major sterol was obtusifoliol. Thus, the 5 environmental isolates described here are similar to the 13 clinical isolates described earlier (8).

In a previous study, we found that relapse *C. neoformans* isolates had a different sterol contents and compositions relative to those of initial isolates recovered before antifungal therapy. Since the initial and relapse isolates had the same RFLP patterns, it appeared that the observed sterol changes occurred in vivo, possibly as a result of selection by the antifungal regimen used or by unknown host factors. In vivo selection that results in altered sterol content and composition of *C. neoformans* could theoretically result in altered susceptibility to amphotericin B, which is believed to interact with fungal membrane sterols to mediate fungicidal effects.

In this study, amphotericin B MIC_{50}s for clinical isolates were higher than those for environmental isolates from the same geographic area but there was no difference in fluconazole susceptibilities. No increases in fluconazole susceptibilities were noted between environmental and clinical isolates. Mouse passage of the five environmental isolates resulted in significant increases in amphotericin B MIC_{50}s for each isolate, and again there was no statistically significant increase in fluconazole MIC_{50}s. Mouse passage was associated with changes in total sterol content and sterol composition for all five passaged isolates. For four of five isolates (M1, B3, B4, and B7), the average ergosterol content increased (from 39.2 to 56.4%) and the average obtusifoliol content decreased (from 42.1 to 23.7%) after passage. For one isolate (B4) there was little

TABLE 4. GLC analysis of sterols of *C. neoformans* isolates pre- and post-murine passage

Sterol	% (wt/wt) of total amt of sterols (mean ± SD) in[a]:									
	M1	M1M	B3	B3M	B4	B4M	B5	B5M	B7	B7M
Squalene	2.8 ± 0.4	5.1 ± 0.1	2.2 ± 0.2	1.7 ± 0.6	1.1 ± 0.4	0.9 ± 0.2	0.7 ± 0.3	0.9 ± 0.4	0.9 ± 0.2	1.8 ± 0.7
Calciferol	11.1 ± 2.5	11.1 ± 0.9	5.1 ± 0.8	9.6 ± 1.0	8.4 ± 6.3	6.1 ± 4.3	2.3 ± 1.0	2.5 ± 1.6	2.5 ± 1.6	4.9 ± 3.8
Zymosterol	0.9 ± 1.2	3.2 ± 4.5	2.7 ± 0.3	ND	ND	ND	ND	0.6 ± 0.8	0.3 ± 0.4	1.3 ± 1.8
Ergosterol	33.0 ± 2.5	40.8 ± 5.1	37.1 ± 2.5	74.1 ± 8.8	52.9 ± 3.0	59.6 ± 4.6	41.7 ± 1.1	41.0 ± 0.9	34.0 ± 2.4	51.3 ± 1.5
Obtusifoliol	41.1 ± 3.1	23.8 ± 1.3	41.0 ± 8.1	5.6 ± 3.8	33.6 ± 8.5	31.4 ± 2.8	46.4 ± 1.3	51.4 ± 8.7	52.8 ± 4.9	34.3 ± 4.7
Lanosterol	10.3 ± 0.2	13.3 ± 2.9	5.6 ± 7.9	8.0 ± 8.1	2.2 ± 3.1	ND	8.9 ± 1.1	3.6 ± 5.1	8.4 ± 1.3	6.4 ± 0.1
24-Methylenedihydrolanosterol	0.9 ± 1.2	2.6 ± 3.7	6.2 ± 1.3	1.0 ± 1.4	1.8 ± 2.5	2.0 ± 2.8	ND	ND	1.1 ± 1.6	ND

[a] Values are from two separate experiments. ND, not detected; suffix M, murine-passaged isolates.

change in either ergosterol or obtusifoliol content. No detectable changes in squalene, calciferol, zymosterol, lanosterol, and 24-methylenedihydrolanosterol content occurred for all strains after passage. Given that each environmental isolate was purified to a single colony twice before murine passage, the observed changes cannot be related to selection from an initial polyclonal environmental isolate used to cause infection. Also, the initial and relapse isolates were stored on Sabouraud dextrose agar slants at 25°C for several months prior to sterol assay. Thus, the changes observed are not the result of temporary alternations in sterol content as an adaptation to the in vivo milieu but are indicative of in vivo selection of new phenotypically stable variants.

Paradoxically, the passaged isolates were more resistant to amphotericin B despite higher ergosterol contents. This result illustrates the complexity of the amphotericin B mechanism of action, which may involve oxidative damage (1) in addition to its effects on membrane sterols and enhancement of macrophage function (17). The global changes in sterol composition may have rendered the murine-passaged strains less susceptible to amphotericin B despite increased ergosterol content. Alternatively, the observed increase in amphotericin B resistance after murine passage may be related not to the apparently paradoxical increase in ergosterol content of the cell membrane but to some other factor yet to be identified. Regardless of the mechanisms, the finding that murine passage reduced susceptibility to amphotericin B for all isolates suggests that drug-resistant variants may arise in vivo without exposure to antifungal agents.

The results illustrate the variability of sterol content, composition, and response to in vivo passage and are significant given the clinical reliance on antibiotics which mediate antifungal effects by interfering with sterol metabolism and function. The finding that sterol composition and amphotericin B susceptibility can change with murine passage suggests that similar studies should be extended to other pathogenic fungi.

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