Susceptibilities of Serial Cryptococcus neoformans Isolates from Patients with Recurrent Cryptococcal Meningitis to Amphotericin B and Fluconazole

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Received 9 February 1993/Accepted 7 April 1993

Amphotericin B and fluconazole susceptibilities of 13 Cryptococcus neoformans isolates from five patients with recurrent cryptococcal meningitis were determined. For each patient, serial isolates showed no increase in antibiotic resistance relative to the initial isolate. For these patients, recurrent disease was not due to drug resistance but may reflect changes in immune function and/or poor compliance.

Cryptococcus neoformans is a yeastlike fungus which causes a progressive meningoencephalitis in 5 to 13% of all patients with AIDS (5, 9, 25). In the setting of AIDS, cryptococcal meningitis is incurable and lifelong suppression with antifungal agents is necessary to decrease the likelihood of recurrence (5, 9, 25, 26). In recent years, fluconazole has become widely used for suppression therapy (14, 24). However, a small number of patients relapse despite fluconazole suppression. We have shown that in recurrent cryptococcal meningitis the initial and relapse isolates are clonally related and hence recurrences result from persistence of the original strain and not from infection with a new strain (21).

To determine whether recurrences of cryptococcal meningitis are due to drug resistance, we measured the amphotericin B and fluconazole susceptibilities of 13 C. neoformans isolates from 5 patients with recurrent cryptococcal meningitis. All isolates were recovered from cerebrospinal fluid and were identified as C. neoformans var. neoformans on the basis of growth on CGB agar (10). Patients J9, J11, and J22 were seen at the Bronx Municipal Hospital Center (Jacobi Hospital, Bronx, N.Y.), and patients SB4 and SB6 were seen at University Hospital at Stony Brook, N.Y. For each patient the A strain is the initial isolate and subsequent letters denote the relapse isolates. In all patients the initial isolates were obtained when the diagnosis was made and before antifungal therapy was begun. The initial and relapse isolates of patients J9, J11, SB4, and SB6 have been shown to be clonally related by URA5 and CNRE-1 restriction fragment length polymorphism analysis and electrophoretic karyotyping of intact chromosomes (21). The J22 isolates were studied only by restriction fragment length polymorphism analysis of polymerase chain reaction-amplified URA5 sequences (4) which revealed no differences between J22A and J22B. Four patients had AIDS (J9, J22, SB4, and SB6), and one (J11) had a chronic lymphocytic leukemia and negative serology for human immunodeficiency virus. Figure 1 shows the temporal relationship between antifungal therapy and recovery of the strains from cerebrospinal fluid. All patients had a prolonged asymptomatic period before clinical recurrence, and patients J9, J22, SB4, and SB6 received suppression therapy with fluconazole prior to relapse.

Susceptibility testing was performed at the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio. Amphotericin B and fluconazole susceptibilities were determined by a broth macrodilution method (7, 13, 17). Inocula were determined spectrophotometrically by using a yeast suspension which gave 95% transmittance at 530 nm, which corresponds to 10⁶ cells per ml. The media used for fungal growth in the amphotericin B and fluconazole susceptibility testing were Antibiotic Medium 3 (M-3; Difco Laboratories, Detroit, Mich.) and Synthetic Amino Acid Medium–Fungal (SAAMF; American Bioorganics, North Tonawanda, N.Y.), respectively. Strains were grown at 35°C. The MICs were read at 24 and 48 h and were defined as the lowest drug concentrations exhibiting no visible growth (turbidity) compared with a drug-free control tube. Minimum lethal concentrations (MLC) were determined by removing 0.1-ml aliquots from each MIC tube demonstrating no visible growth and plating to the surface of a Sabouraud dextrose agar plate (Difco). The plate prepared from the MIC tube containing the lowest drug concentration showing ≤5 colonies was considered to have the MLC. All tests were done in duplicate with well-characterized strains as controls (17). The antifungal susceptibilities for each set of isolates were performed on the same day to ensure comparability of MIC data within a series.

Amphotericin B and fluconazole susceptibility data for the 13 isolates are shown in Table 1. MICs of amphotericin B for the initial isolates, measured at 24 and 48 h, all fell within the narrow range of ≤0.14 to 0.29 μg/ml. MICs at 24 h were all ≤0.29 μg/ml, and at 48 h, the MLCs for 10 of 13 strains were ≤0.58 μg/ml, consistent with the known fungicidal activity of amphotericin B.

MICs of fluconazole at 24 h ranged from ≤0.125 to 20 μg/ml. Despite this wide range, there was no increase in MIC among serial isolates from individual patients. For one patient (J9) there was actually a decrease in the MICs for the relapse isolates relative to the initial isolate (see below). MICs measured at 48 h were generally higher and more narrowly distributed and had no significant increases among serial isolates. In 7 of 13 strains there was a four- to eightfold

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increase in MIC between the 24- and 48-h readings. Fluconazole exhibited little or no fungicidal activity except in two isolates from one patient (J9C and J9D).

Recent studies on the development of standardized antifungal susceptibility assays have specifically noted difficulties with *C. neoformans* susceptibility testing (6, 8, 13). Unresolved questions include the optimum length of incubation and the definition of the endpoint. The differences in MICs at 24 versus 48 h for some of our strains illustrate the difficulties in *C. neoformans* susceptibility testing. The paucity of data correlating MICs with in vivo outcome has also prevented the establishment of guidelines for interpreting fluconazole MICs. MICs of amphotericin B and fluconazole for all of the isolates were lower than the achievable levels in

FIG. 1. Clinical course of patients J9, J11, J22, SB4, and SB6 showing the temporal relationship between the initial and relapse isolates, duration and type of antifungal therapy, and the antigen titers in cerebrospinal fluid at the various time points. Hatched lines indicate periods of hospitalization. Patients SB4 and SB6 received intravenous fluconazole during the initial hospitalization. AMP B, amphotericin B; 5-FC, 5-flucytosine. Fluconazole suppression doses were 400 mg/day for each patient, with the exception of J9, for whom 200 mg/day was prescribed prior to the January hospitalization.
TABLE 1. Results of in vitro antifungal susceptibility tests for the initial and relapse C. neoformans isolates

| Strain | Amphotericin B | | | Fluconazole | | |
|--------|----------------|-----------------|-----------------|---------------------|---------------------|
|        | MIC (µg/ml) 24 h | MLC (µg/ml) 24 h | MIC (µg/ml) 24 h | MLC (µg/ml) 24 h |
| J9A    | ≤0.14          | ≤0.14           | ≤0.14           | ≤0.14 | 0.58 |
| J9B    | 0.29           | 0.29            | 0.29            | 0.29 | 0.58 |
| J9C    | 0.29           | 0.29            | 0.29            | 0.29 | 0.29 |
| J9D    | 0.29           | 0.29            | 0.29            | 0.29 | 0.29 |
| J11A   | ≤0.14          | 0.29            | 0.29            | 0.29 | 1.16 |
| J11B   | ≤0.14          | 0.29            | 0.29            | 0.29 | 0.58 |
| J22A   | ≤0.14          | 0.29            | 0.29            | 0.29 | 2.31 |
| J22B   | ≤0.14          | 0.29            | 0.29            | 0.29 | 0.58 |
| SB4A   | ≤0.14          | 0.29            | 0.29            | 0.29 | 0.58 |
| SB4B   | ≤0.14          | 0.29            | 0.29            | 0.29 | 0.58 |
| SB4C   | ≤0.14          | 0.29            | 0.29            | 0.29 | 0.58 |
| SB6A   | ≤0.14          | 0.29            | 0.29            | 0.29 | 2.31 |
| SB6B   | ≤0.14          | 0.29            | 0.29            | 0.29 | 0.58 |

serum for these drugs (1, 3, 16). For the four AIDS patients on fluconazole suppression (J9, J22, SB4, and SB6) the MICs and MLCs for the relapse isolates were the same as or lower than those for the initial isolates at 24 h. Thus, the MIC data from our isolates provide strong evidence against fluconazole resistance being the cause of recurrent cryptococcal meningitis. Surprisingly, the MICs of fluconazole for J9 isolates exhibited a decrease despite long-term therapy with this drug. Although the basis for this increased susceptibility is not known, the MIC results for the J9 strains are consistent with the long asymptomatic period experienced by this patient following recurrence of his meningitis and treatment with amphotericin B.

There has been one well-documented case of amphotericin B-resistant C. neoformans in a patient with AIDS (15). Amphotericin B is a polyene antibiotic which mediates antifungal effects by binding to cell membrane sterols, damaging the cell membrane and resulting in leakage of essential metabolites (3). Fluconazole is a triazole which inhibits membrane sterol synthesis (18). Since azoles block the synthesis of ergosterol, the target of amphotericin B, there has been concern about using these two types of antifungal agents together (2, 19, 20, 22, 23). C. albicans (23) and Aspergillus fumigatus (20) have been reported to become resistant to amphotericin B in the presence of ketoconazole (23). In this regard it is noteworthy that chronic fluconazole suppression in our patient group did not increase C. neoformans resistance to amphotericin B.

The potential development of fluconazole resistance is a serious concern, given the widespread use of fluconazole suppression therapy. Our data indicate that fluconazole MICs can vary widely among isolates; therefore, it is essential to test paired isolates from the same patient when investigating relapses of cryptococcal meningitis. Where there is an increase in MIC, it is important to type strains to distinguish between selection for resistant mutants versus the acquisition of new resistant strains. This is now possible for many fungi, including C. neoformans, given the development of molecular probes for genetic typing.

Recurrent cryptococcal meningitis results from persistence of the initial infection despite antifungal therapy (21). Our finding that the initial and relapse C. neoformans isolates are susceptible to amphotericin B and fluconazole in vitro strongly suggests that recurrences are not due to drug resistance. Recurrences of cryptococcal meningitis may reflect either poor compliance with the suppression drug regimen or further deterioration of the immune system. New therapeutic approaches for the treatment of C. neoformans infections are urgently needed. Potential strategies to improve therapy include combinations of antifungal drugs and/or use of immunomodulators such as anti-cryptococcal antibodies (11, 12) to enhance the ability of the host to clear the infection.

(Revised the fluconazole data in this paper have been accepted as an abstract to the American Society for Microbiology General Meeting in May 1993.)

A.C. was supported in part by a Pfizer Postdoctoral Fellowship and a James S. McDonnell award. E.D.S. is supported by PHS grant AI52340 from the NIH.

The excellent laboratory skill of D. A. McGough and A. W. Fothergill is gratefully acknowledged. We thank Silvia G. Spitzer for the polymerase chain reaction analysis of the J22 isolates.

REFERENCES

NOTES


