Correlation of Susceptibility of *Cryptococcus neoformans* to Amphotericin B with Clinical Outcome


Department of Medicine (Infectious Diseases), University of Southern California, Los Angeles, California; Faculty of Tropical Medicine, Mahidol University, Bangkok, and Bamrasnardura Hospital, Communicable Disease Control Department, Nonthaburi, Thailand; Department of Pathology, University of Texas Health Science Center of San Antonio, San Antonio, Texas; Instituto de Medicina Tropical Alexander Von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru; University of Northern Colorado, Greeley, Colorado; and Centre for Tropical Medicine, Naufield Department of Clinical Medicine, University of Oxford, Churchill Hospital, Oxford, United Kingdom

Received 10 January 2011/Returned for modification 15 April 2011/Accepted 11 September 2011

Testing of *Cryptococcus neoformans* for susceptibility to antifungal drugs by standard microtiter methods has not been shown to correlate with clinical outcomes. This report describes a modified quantitative broth macrodilution susceptibility method showing a correlation with both the patient’s quantitative biological response in the cerebrospinal fluid (CSF) and the survival of 85 patients treated with amphotericin B (AMB). The Spearman rank correlation between the quantitative in vitro measure of susceptibility and the quantitative measure of the number of organisms in the patient’s CSF was 0.37 (P < 0.01; 95% confidence interval [95% CI], 0.20, 0.60) for the first susceptibility test replicate and 0.46 (P < 0.001; 95% CI, 0.21, 0.62) for the second susceptibility test replicate. The median in vitro estimated response (defined as the fungal burden after AMB treatment) at 1.5 mg/liter AMB for patients alive at day 14 was 5 CFU (95% CI, 3, 8), compared to 57 CFU (95% CI, 4, 832) for those who died before day 14. These exploratory results suggest that patients whose isolates show a quantitative in vitro susceptibility response below 10 CFU/ml were more likely to survive beyond day 14.

A reliable and reproducible method for estimating biological responses to antifungal drugs in cryptococcal meningitis remains elusive. A method of drug susceptibility testing for yeasts has been proposed, but no interpretive guidelines for *Cryptococcus neoformans* have been validated. Standard methods for testing susceptibility to amphotericin B (AMB) provide epidemiological cutoff values (ECV) that have not been shown to correlate with therapeutic responses or subsequent survival. We described previously a modified broth macrodilution method for which there was a strong correlation between the quantitative measure of susceptibility to AMB in vitro and the quantitative biological response observed in AIDS patients with cryptococcal meningitis. Those findings have now been replicated using this method of AMB susceptibility testing for an independent cohort of AIDS patients with cryptococcal meningitis who were treated with AMB.

(This work was presented in part at the 7th International Conference on Cryptococcus & Cryptococcosis, Nagasaki, Japan, September 2008.)

*MATERIALS AND METHODS*

**Patients.** All patients had AIDS-associated cryptococcal meningitis and were treated with AMB at 0.7 mg/kg of body weight/day for 14 days in a clinical study conducted in Thailand as described previously in detail (13). A total of 110 patients were enrolled in the clinical trial. Routine lumbar punctures were performed; quantitative yeast counts were performed; and the cerebrospinal fluid (CSF) was cultured on Sabouraud agar plates as described previously (13). Cryptococcal isolates from 85 patients were available for testing of susceptibility to AMB. The baseline isolate did not survive storage for 19 patients, and 6 patients had no follow-up information to allow evaluation of the response to treatment. The intensity of meningeal infection was assessed at baseline by quantitative culture taken directly from the test tube after incubation for 48 h rather than by visual assessment or spectrophotometric measurement of turbidity. We used a target inoculum that matched the number of organisms present in the CSF sample. CSF samples were collected for cryptococcal counts and culture at weekly intervals for at least 2 weeks or until CSF cultures were negative in order to assess the response to treatment. Ethical review board approval was obtained from the scientific review subcommittee of the Thai Ministry of Public Health for the conduct of the clinical study and from the Human Subjects Review Board at the University of Southern California for the susceptibility testing.

**Mycology.** The *C. neoformans* isolates were recovered from the CSF before treatment, cultured, stored at ~70°C in 10% glycerol, shipped on dry ice, revived, and stored in skim milk at ~20°C until use. By use of the standard CLSI (formerly NCCLS) microtiter method, the MICs of AMB were measured in triplicate employing inocula between 500 and 2,500 CFU/ml. In addition to testing with RPMI medium per the CLSI standard, isolates were also tested with M3 medium, since it has been suggested that the latter medium would expand the range of AMB susceptibility and thus allow for more-sensitive identification of resistant isolates. The MIC for each isolate was expressed as the median value of the three replicates.

The broth macrodilution method was modeled after the standard CLSI M27-A2 macrobroth assay (9). The modified broth macrodilution method employed a target inoculum that matched the number of organisms present in the CSF at presentation for each individual patient, rather than using a fixed inoculum of 500 to 2,500 CFU/ml. The endpoint was determined by quantitative culture taken directly from the test tube after incubation for 48 h rather than by visual assessment or spectrophotometric measurement of turbidity. We used

* Corresponding author. Mailing address: Department of Medicine (Infectious Diseases), LAC+USC Medical Center, IRD Building, 2020 Zonal Ave., Room 430, MC 39220, Los Angeles, CA 90033. Phone: (323) 226-7504. Fax: (323) 226-2450. E-mail: rlarsen@usc.edu.

† Published ahead of print on 26 September 2011.

‡ The authors have paid a fee to allow immediate free access to this article.
drug concentrations spanning a range determined by experiments to include concentrations that produced in vitro responses matching each patient’s quantitative response (8), rather than using drug levels predicted to be achievable in patients. The exact inoculum was confirmed by quantitative culture, and susceptibility was retested if the inoculum was not within 0.5 log10 unit of the target. Susceptibility for each isolate was measured on at least two separate occasions by use of this modified broth macrodilution method. The susceptibility test was repeated if the 99% confidence intervals (CIs) for the concentration-response curves of the replicates for a given isolate did not overlap or if the maximum CI width (difference between the upper limit and the lower limit) was greater than 2 log10 units. A positive-control isolate (USC 1597) was tested weekly to ensure AMB drug potency. The AMB concentrations tested ranged from 0 to 3.5 mg/liter.

**Statistical analysis.** Local nonparametric regression (Loess regression) was used to estimate the AMB concentration-response curve and to determine the 99% CIs for each isolate (3, 4). Loess regression is particularly appropriate for susceptibility testing, since it is biologically reasonable that there is a smooth gradual change in response as the dose concentration is increased or decreased slightly. Since the Loess method uses local weighted regression to estimate the response at each dose concentration, changes can be detected in the patterns of association between response and drug concentration across the range of concentrations. For each isolate, the predicted response was based on the estimated in vitro response at AMB at a concentration of 1.5 mg/liter (6). Due to the exploratory nature of these analyses, Spearman’s rank order statistic (rho, a nonparametric measure based on ranks) was used to evaluate the association between the quantitative in vitro response predicted from Loess analysis and the patient’s quantitative response at day 14 (7). “Box-and-whisker” plots (box plots) were used to provide a visual display of the distribution of quantitative variables (e.g., the patient’s pretreatment CSF fungal burden) for various groups. For each group, the “box” showed the range of the middle 50% of observations of the quantitative variable, such as the patient’s pretreatment CSF fungal burden. The filled line in each box was drawn at the median value of the quantitative variable for that group; the shaded area represented the 95% CI for the median. The amount by which the CIs overlapped or did not overlap provided a visual exploratory evaluation of the differences in the distributions of the quantitative variable among the groups. The S-Plus statistical system was used for all statistical analyses and graphical displays (16, 18).

**RESULTS**

**Baseline characteristics.** The severity of meningitis, as measured by quantitative C. neoformans counts in the pretreatment CSF samples, ranged from 100 to 9,200,000 CFU/ml, with a median value of 37,700 (95% CI, 25,100 to 50,100) CFU/ml for the 85 patients whose isolates were tested. For the 19 patients whose isolates were not tested for susceptibility, the median CSF yeast burden was 39,800 (95% CI, 10,000 to 158,500) CFU/ml. Of the 85 patients whose isolates were tested, 64 patients had a quantitative biological response assessed at day 14, 11 died before day 14, and 10 did not have a day 14 CSF sample collected. There were no apparent differences among these groups in the distribution of the pretreatment severity of meningitis (Fig. 1).

**Susceptibility by the modified broth macrodilution method.** Figure 2 shows the detailed in vitro AMB concentration-response curves for each of the 64 patients from whom isolates were collected and for whom an associated quantitative biological response (defined as the fungal burden, in CFU per milliliter, after AMB treatment) was assessed at day 14. The Loess regression used a span (smoothing parameter) of 1.0 and quadratic local regression (degree, 2). The point on each in vitro concentration-response curve where the in vitro response matched the patient’s observed quantitative biological response at day 14 provides an estimate of the equivalent in vitro AMB concentration. The equivalent in vitro AMB concentration was undefined for two isolates for which the observed patient responses were lower than the in vitro responses at the highest AMB concentration tested.

In our previous study of the association between quantitative patient responses at day 14 and in vitro responses to AMB, the median estimated equivalent AMB concentration was 1.5 mg/liter (95% CI, 1.3, 1.7) (8). In the present study, the median estimated equivalent AMB concentration was 1.25 mg/liter (95% CI, 1.15, 1.35) in the first replicate and 1.0 mg/liter (95% CI, 0.91, 1.09) in the second replicate. Although the CIs did not overlap, the Spearman rank correlation between the in vitro estimated response at 1.5 mg/liter AMB based on the estimated concentration-response curve and the patient’s observed quantitative biological response at day 14 was 0.37 (P < 0.01; 95% CI, 0.20, 0.60) for the first replicate and 0.46 (P < 0.001; 95% CI, 0.21, 0.62) for the second replicate (Fig. 3A). The Spearman rank correlation between the measured in vitro CFU count in the single broth macrodilution tube tested at an AMB concentration of 1.5 mg/liter and the associated observed quantitative biological response at day 14 was 0.31 (P < 0.02; 95% CI, 0.15, 0.56) for the first replicate and 0.37 (P < 0.005; 95% CI, 0.15, 0.58) for the second replicate.

**Susceptibility by the standard microtiter method.** A total of 85 isolates were tested using the modified macrobroth method, and 84 isolates were tested using the microtiter method. The one isolate that was not tested using the microtiter method did not survive shipping to the reference lab where the microtiter MICs were measured. Of the 84 isolates tested by the microtiter method, 44 were tested in duplicate or triplicate using the standard microtiter method. The MICs for 100% of these 44 isolates were within 2 dilutions; the median of the replicates was used as the MIC for each isolate (Table 1). The AMB MICs by the microtiter method at 72 h were between 0.06 and 2 mg/liter with RPMI medium and between 0.06 and 1 mg/liter with M3 medium. Although the use of M3 medium did not expand the range of the MICs, the MICs with RPMI medium were within 2 dilutions of the MICs with M3 medium for all isolates. Figure 3B shows the distributions of the patients’ observed quantitative biological responses at day 14 versus the
FIG. 2. Loess fit of the AMB concentration-response curves for isolates from patients treated with AMB alone. The susceptibility of each isolate was measured twice (replicates 1 and 2). The filled circle placed at an AMB concentration of 1.5 mg/liter shows the level of the patient’s quantitative biological response at day 14 (in log₁₀ CFU/ml). The shaded vertical bar above each patient’s data indicates the relative baseline fungal burden (in CFU/ml of CSF). The data are ordered by baseline fungal burden, starting at the bottom left of panel A (500 CFU/ml) and increasing from left to right and from bottom to top to the top right of panel B (1,400,000 CFU/ml).
FIG. 2—Continued.

B. Numbers of CFU (Log$_{10}$/mL) at 48 Hours

AMB Concentration (mg/L)

Day 14 Quantitative Biological Response (Log$_{10}$/CFU/mL)

Day 14 Response

Replicate 1

Replicate 2
72-h microtiter MICs of AMB with RPMI medium. The 95% CIs for the median biological response at day 14 overlap for all levels of the 72-h microtiter MICs of AMB, suggesting that there was no apparent association between the *in vitro* microtiter susceptibility testing result and the patient’s biological response.

**Association between susceptibility and survival.** Table 1 shows the proportions of patients surviving at days 14 and 28 for each level of the 72-h microtiter MIC of AMB. The association between survival at day 14 and susceptibility to AMB based on the microtiter method was significant (P < 0.03), but the association with day 28 survival was not significant (P = 0.09). Figure 4a shows the association between survival at day 14 and *in vitro* susceptibility measured by the modified macrobroth method. For patients alive at day 14, the median *in vitro* estimated response at 1.5 mg/liter AMB was 5 CFU (95% CI, 3, 8), compared to 57 CFU (95% CI, 3, 832) for those who died before day 14. These exploratory results suggest that patients whose isolates showed an *in vitro* susceptibility response below 10 CFU/ml were more likely to survive beyond day 14. Figure 4B shows the association between survival at day 28 and *in vitro* susceptibility measured by the modified macrobroth method. For patients alive at day 28, the median *in vitro* estimated response at 1.5 mg/liter AMB was 5 CFU (95% CI, 3, 8), compared to 16 CFU (95% CI, 3, 79) for those who died before day 28. These exploratory results also suggest that patients whose isolates showed an *in vitro* susceptibility result below 10 CFU/ml were more likely to survive beyond day 28.

**DISCUSSION**

In clinical practice, antimicrobial susceptibility testing is performed to predict the drug(s) most likely to provide the optimal clinical outcome. However, efforts to correlate the susceptibility of *C. neoformans* to AMB with clinical outcome have been hampered by the narrow range of susceptibility of this organism when it is tested using current “standard” methods. These methods employ an inoculum corresponding to the pretreatment fungal burden associated with only the mildest cryptococcal meningitis. The single most important feature associated with clinical outcome in patients with AIDS-associated cryptococcal meningitis is the pretreatment severity of meningitis (15). The incorporation of a measure of the initial severity of meningitis into testing of the susceptibility of isolates to antifungal drugs provided a more consistent and more clinically relevant picture of the anticipated microbiological and clinical responses of patients with AIDS-associated cryptococcal meningitis treated with AMB at a dose of 0.7 mg/kg given daily (8).

The MICs based on the “standard” microtiter method of antifungal drug susceptibility testing were within 1 dilution for 91% of the *Cryptococcus neoformans* var. *neoformans* isolates and did not correlate with the patients’ observed quantitative biological responses at day 14. Despite the lack of association between MIC values and observed patient responses in the CSF at day 14, there was an association between the CLSI microtiter MIC values and patient survival at day 14 and a

**TABLE 1. Association between isolate susceptibility to AMB by the microtiter method and patient survival**

<table>
<thead>
<tr>
<th>Day and medium</th>
<th>No. of patients alive/total no. with isolates with the following AMB MIC (mg/liter) at 72 h:</th>
<th>(p^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPMI</td>
<td>0.06 0.125 0.25 0.5 1.0 2.0</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>0.06 0.125 0.25 0.5 1.0 2.0</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPMI</td>
<td>0.06 0.125 0.25 0.5 1.0 2.0</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>0.06 0.125 0.25 0.5 1.0 2.0</td>
<td></td>
</tr>
</tbody>
</table>

\(a^a\) By Fisher’s exact test.
suggestion of improved survival at day 28. These results are intriguing and suggest that a general measure of *in vitro* susceptibility using the CLSI microtiter method may have predictive value. The lack of association between the microtiter method and patient mycological responses at day 14, as well as the small numbers of patients at each given AMB MIC value, make the association tenuous, but worthy of additional investigation.

Any modification of the microtiter method to incorporate a measure of the initial severity of meningitis would yield confounded results, because the endpoint determination of the microtiter method relies on turbidity. Fungal burdens that exceed approximately 50,000 CFU/ml give cloudy microtiter wells that obscure the endpoint. In contrast to bacterial testing, the killed *C. neoformans* is not lysed. Thus, wells remain turbid even at drug concentrations that kill the organism. For the majority of patients with AIDS-associated cryptococcal meningitis, the initial burden of organisms in the CSF often far exceeds 50,000 CFU/ml. Furthermore, efforts to alter the microtiter methodology to incorporate quantitative assessments of susceptibility fail because the small sample volume and the difficulty of manipulating microtiter wells preclude efficient and reliable sampling of the wells to assess the presence of viable organisms remaining after AMB exposure.

The modified broth macrodilution method for susceptibility testing reported here has the potential to provide clinicians with valuable information regarding the anticipated clinical response to treatment. The results can be made available to the clinician within a 7-day period, a time window within which important clinical decisions can be made regarding changes in antifungal therapy (10). If *in vitro* antifungal drug susceptibility testing suggests that large numbers of *C. neoformans* organisms are likely to remain in the CSF after 14 days of treatment with AMB, it may be appropriate to extend treatment with AMB longer than 14 days, to increase the dose of AMB (1), and/or to combine AMB with either flucytosine or fluconazole (2, 17), in hopes of achieving an improved biological response. If few or no *C. neoformans* organisms are predicted to remain on the basis of susceptibility testing, this prediction provides confidence that switching from an AMB-based treatment regimen to fluconazole therapy at 14 days will ultimately be successful. It is noteworthy that patients with negative CSF cultures after 14 days of AMB treatment are 4- to 5-fold more likely to survive for ≥10 weeks (15, 17).

Although it is not common practice to quantify the number of organisms in the CSF of persons with cryptococcal meningitis either at baseline or during follow-up, quantitative cultures are simple and inexpensive to perform and are far more clinically informative than qualitative responses once treatment has begun. We believe microbiology laboratories should routinely adopt the practice of performing quantitative CSF counts and cultures for patients with cryptococcal meningitis. Valuable clinical information is lost by simply reporting qualitative (positive or negative) results. Furthermore, in the absence of a quantitative CSF culture, it is not possible to estimate the appropriate inoculum for susceptibility testing.

The clinical significance of small numbers of organisms persisting either in the CSF or in the *in vitro* susceptibility macrobroth test needs further investigation. Nearly all previous reports have used qualitative rather than quantitative CSF cultures, thus providing little information to guide clinicians on the significance of small numbers of cryptococcal organisms remaining after 2 weeks of treatment. In such qualitative reports, the presence of a single organism is not distinguished from the presence of 10,000 CFU or more. The method of analysis that we have employed capitalizes on the richer information provided by the concentration-response curve estimated over a range of concentrations of AMB to estimate the response at the AMB concentration of 1.5 mg/liter. Assessment at only a single AMB concentration is more prone to error. However, in our study, the *in vitro* response measured in a single test tube at 1.5 mg of AMB/liter still had a good correlation with the patient’s day 14 quantitative biological response (*P* < 0.02). Thus, this simpler assessment has the potential to be readily adapted to routine clinical practice. Further investigation with additional isolates from patients...
treated with AMB alone is needed in order to develop a simplified assay and evaluate the additional predictive value in relation to patient characteristics such as pretreatment CSF fungal burden and measures of HIV status.

The reagents for testing the susceptibility of isolates to AMB are readily available, but the preparation of the AMB concentrations used in susceptibility testing does require careful attention, and we have found it necessary to include a drug control test isolate with each susceptibility run to monitor the potency of AMB from run to run as a quality assurance measure.

This study shows that appropriately designed testing of susceptibility to antifungal drugs in vitro can provide useful information regarding the potential therapeutic responses of cryptococcal meningitis to AMB treatment. It is evident that concentration-response relationships differ substantially and that patients with high pretreatment CSF fungal burdens will commonly require either higher doses of AMB, extended treatment courses, treatment with a combination of AMB and other agents (fluconazole or flucytosine), or all three approaches for optimum treatment.

ACKNOWLEDGMENTS

We thank our student volunteers, Anna K. Celaya, Alexis Korostoff, and Rachel Buchmeier, for their enthusiasm and assistance in the laboratory. We are grateful for the support and assistance given by the director and staff of Bamrasonaradura Hospital and the Mahidol-Oxford Tropical Medicine Research Unit.

This work was supported in part by grants from the National Institute of Allergy and Infectious Diseases (N01-AI-070091) and the Infectious Diseases Society of America, and was part of the Wellcome Trust Mahidol University-Oxford Tropical Medicine Research Programme, funded by the Wellcome Trust, United Kingdom.

REFERENCES