Resistance to amphotericin B does not emerge during treatment for invasive aspergillosis

Mahomed-Yunus S. Moosa, George J. Alangaden, Elias Manavathu and Pranatharthi H. Chandrasekar*

Division of Infectious Diseases, Department of Internal Medicine, Wayne State University School of Medicine, Detroit, MI 48201, USA

Emergence of resistance to antifungal drugs during therapy for invasive aspergillosis has received scant attention. We recovered Aspergillus isolates from six patients with invasive aspergillosis, who were receiving amphotericin B before fungal isolation. Although isolates were susceptible to amphotericin B in vitro, none of the patients survived. The MIC of amphotericin B for isolates was similar to that for isolates from 35 patients with no prior exposure to amphotericin B. Laboratory attempts to produce amphotericin B resistance in Aspergillus were unsuccessful. These data indicate that emergence of resistance to amphotericin B is uncommon during therapy for invasive aspergillosis.

Introduction

The incidence of invasive aspergillosis has substantially increased in the last two decades. Over the years amphotericin B has become the standard treatment. Overall, the response to amphotericin B remains poor, with a favourable outcome in only 30–40% of treated patients. The exact reasons for such a dismal outcome remain unclear. It is widely believed that therapeutic failure is closely associated with diagnostic delay as well as poor immune status of the host. Intriguingly, Lass-Flörl et al.1 proposed that resistance of the fungus to amphotericin B or poor pharmacokinetics of the drug might play a role in the dismal response. The potential for Aspergillus fumigatus to develop resistance to amphotericin B was demonstrated by our finding that ultraviolet irradiation of germinating conidia enhanced the selection of amphotericin B-resistant mutants.2 Furthermore, Seo et al.3 reported the generation of polyene-resistant mutants following the repeated passage of Aspergillus flavus in graded concentrations of amphotericin B. These findings indicate that increased clinical use of amphotericin B may select for resistant Aspergillus isolates, possibly contributing to the poor outcome in invasive aspergillosis.

To address the question of prolonged antifungal therapy contributing to resistance emergence in Aspergillus, we recovered six isolates from patients receiving amphotericin B for invasive aspergillosis. These isolates were characterized and tested for resistance to amphotericin B in vitro. The results were reviewed in the context of the clinical profile of these patients and the final outcome. For comparison, we tested the antifungal susceptibility of Aspergillus isolates obtained from patients with no prior antifungal exposure. We also examined the possibility of selecting amphotericin B-resistant mutants by passaging Aspergillus isolates on plates containing various concentrations of the drug.

Materials and methods

Patients

We identified six patients with fatal invasive aspergillosis treated at Harper Hospital, Wayne State University School of Medicine between 1996 and 1999. Three had culture-proven aspergillosis before treatment, whereas the remaining three were treated based on characteristic clinical and radiological findings. All six isolates available for testing were obtained from the six patients after they had received amphotericin B treatment for a variable duration.

*Correspondence address. Division of Infectious Diseases, 4 Yellow Center, Room 415, Harper Hospital, 3990 John R, Detroit, MI 48201, USA. Tel: +1-313-745-9649; Fax: +1-313-993-0302; E-mail: pchandrasekar@intmed.wayne.edu

© 2002 The British Society for Antimicrobial Chemotherapy
Aspergillus isolates

The clinical isolates of *A. fumigatus* and *Aspergillus niger* used in this investigation were isolated from bronchial wash fluid and sputum at the Microbiology Laboratory of the Detroit Medical Center, Detroit, MI, USA. Working cultures of the isolates were maintained on Sabouraud dextrose agar slants at 4°C. Thirty-five *Aspergillus* isolates from patients with no prior exposure to any antifungal treatment were also examined (controls).

Antifungal drugs tested

The antifungal agents used in this study were obtained as pure powders from the manufacturers. Itraconazole (R51 211, batch no. STAN-9304-005-1) was obtained from Janssen Pharmaceutica, Beerse, Belgium; voriconazole (batch no. 25381-57-8) from Pfizer Inc., New York, NY, USA; amphotericin B (batch no. 20-914-29670) from Squibb Institute for Medical Research, Princeton, NJ, USA; and posaconazole (batch no. 97-56592-X-208) from Schering-Plough Research Institute, Kenilworth, NJ, USA. All antifungals were dissolved in dimethyl sulfoxide at a concentration of 1 g/L and stored at −20°C. The frozen stock was thawed at room temperature and gently vortexed before use. The concentrations of various drugs used for MIC studies ranged from 0.0625 to 16 mg/L. Where applicable, comparable concentrations of dimethyl sulfoxide were used as solvent controls.

MIC determination

The in vitro susceptibilities of various isolates of *Aspergillus* species to antifungal agents were determined by a broth microdilution method similar to the NCCLS M38-P protocol using peptone yeast extract glucose (PYG) medium [Bacto-peptone (Difco Chemicals, Detroit, MI, USA), 1 g yeast extract, 1 g glucose, 3 g/L distilled water], since RPMI 1640 is unsuitable for detecting amphotericin B resistance. Briefly, fresh conidia were collected from various *Aspergillus* isolates and suspended in PYG medium at a density of 2 × 10⁴ conidia/mL. Twice the required concentration of the drugs was prepared in the same medium (0.1 mL) by serial dilution in 96-well round bottom microtitre plates (Rainin Instruments Company, Woburn, MA, USA) and inoculated with an equal volume (0.1 mL) of the conidial suspension. The microtitre plates were incubated at 35°C for 48 h and scored for visible growth with the aid of a viewing mirror. The MIC, defined as the lowest concentration of drug that produces no visible growth, was determined in duplicate with the experiment repeated once.

In vitro resistance selection

Conidial suspensions prepared from a susceptible clinical isolate of *A. fumigatus* (ATCC 208966) were plated (1 × 10⁶ conidia/plate) on PYG agar containing amphotericin B (8–16 mg/mL) and incubated at 35°C for 6 days. Colonies that grew on plates containing amphotericin B were further tested for resistance by in vitro susceptibility testing.

Results

All six patients in this study were profoundly immunosuppressed (Table 1). Three patients were bone marrow transplant recipients on high dose corticosteroids for severe graft-versus-host disease (two patients) or radiation pneumonitis (one patient); furthermore, two of these three patients (patients 1 and 4) also had graft failure or hypofunction. One patient had advanced AIDS. Two patients were neutropenic, receiving chemotherapy for haematological malignancies (acute myeloid leukaemia, high-grade non-Hodgkin’s lymphoma) at the time of diagnosis.

All isolates were *A. fumigatus* with the exception of one (*A. niger*). Duration of therapy before isolation of the *Aspergillus* ranged from 7 to 72 days (mean 24 days, median 17 days). All patients received either amphotericin B or a liposomal derivative. In addition, patients 3 and 4 received growth factors (GM-CSF), and patient 5 received itraconazole for at least 1 month. All patients died 12–73 days (mean 33 days, median 30 days) after diagnosis of invasive aspergillosis.

All six *Aspergillus* isolates were susceptible to amphotericin B; MICs ranged from 0.125 to 0.5 mg/L. The range of MICs of amphotericin B for *Aspergillus* isolates from 35 patients with no prior exposure to the antifungal agent was similar (Table 2). All isolates, from patients and controls, were equally susceptible to itraconazole and the newer triazoles voriconazole and posaconazole (Table 2).

In the laboratory, spontaneous mutants of *A. fumigatus* with decreased susceptibility to amphotericin B could not be selected on PYG agar (c. 500 plates with 1 × 10⁶ conidia/plate) containing 8–16 mg/mL amphotericin B. Colonies that grew (frequency c. 5 × 10⁻⁸) on amphotericin B-containing plates did not demonstrate reduced susceptibility on further testing.

Discussion

Emergence of resistance to amphotericin B during therapy for invasive aspergillosis was not encountered in this study. The MICs of various drugs for all six isolates under study were <1 mg/L, within the range considered susceptible for all antifungals tested, and no different from the MICs of amphotericin B for control isolates recovered from amphotericin B-naïve patients. Furthermore, passaging *Aspergillus* isolates in the presence of amphotericin B failed to select for mutants with increased MICs. Previous attempts to select for spontaneous amphotericin B-resistant mutants had failed to yield any isolate that maintained this phenotype on repeated passaging. These findings indicate that
<table>
<thead>
<tr>
<th>Organism</th>
<th>Underlying disease</th>
<th>Drug</th>
<th>Dose</th>
<th>Duration of treatment before fungal isolation (days)</th>
<th>Site of disease</th>
<th>Nature of immune suppression</th>
<th>Time from initiation of treatment to death (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. fumigatus</em></td>
<td>aplastic anaemia</td>
<td>ABCD</td>
<td>6 mg/kg</td>
<td>10</td>
<td>lung, sinus</td>
<td>neutropenia, graft failure, high dose steroids, ATG</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>PNH</td>
<td></td>
<td></td>
<td></td>
<td>brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BMT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>AML</td>
<td>ABLC</td>
<td>5 mg/kg</td>
<td>7</td>
<td>lungs, brain, thyroid, subcutaneous mass</td>
<td>neutropenia, chemotherapy</td>
<td>30</td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>ALL</td>
<td>ABLC</td>
<td>0.7 mg/kg</td>
<td>11</td>
<td>lung</td>
<td>high dose steroids, graft failure</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>BMT</td>
<td>ABLC</td>
<td>5 mg/kg</td>
<td></td>
<td>brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GM-CSF</td>
<td>ABLC</td>
<td>5 μg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>CLL</td>
<td>ABCD</td>
<td>6 mg/kg</td>
<td>17</td>
<td>lung</td>
<td>high dose steroids, graft failure</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>2° PLL</td>
<td>GM-CSF</td>
<td>5 μg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>AIDS</td>
<td>AMB</td>
<td>0.7 mg/kg</td>
<td>72</td>
<td>lung</td>
<td>CD4 &lt; 50, V_L = 200 000/μL</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>itraconazole</td>
<td>200 mg bd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABLC</td>
<td>5 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>Walden.</td>
<td>AMB</td>
<td>0.7 mg/kg</td>
<td>29</td>
<td>lung</td>
<td>active high grade NHL, neutropenia, chemotherapy, neutropenia</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>brain</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ABCD, amphotericin B colloidal dispersion; ABLC, amphotericin B lipid complex; AMB, amphotericin B; ATG, anti-thymocyte globulin; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; BMT, bone marrow transplant; CLL, chronic lymphatic leukaemia; GM-CSF, granulocyte–macrophage colony stimulating factor; AIDS, acquired immune deficiency syndrome; NHL, non-Hodgkin’s lymphoma; PNH, paroxysmal nocturnal haemoglobinuria; PLL, prolymphocytic leukaemia; V_L, HIV viral load; Walden., Waldenstrom’s macroglobulinaemia.
exposure to amphotericin B does not select for resistant mutants, hence downplaying the contribution of secondary resistance to the uniformly poor outcome of invasive aspergillosis. However, the relevance of in vitro amphotericin B susceptibility testing as a predictor of clinical outcome remains controversial. Whereas Lass-Flörl et al.,1 using data from human subjects, have demonstrated a clear correlation between in vitro susceptibility to amphotericin B and clinical outcome of invasive aspergillosis, others,5–7 using animal models, have shown a poor correlation.

Amphotericin B has been used to treat fungal infections for over 40 years. Despite this long period of use, clinical resistance among Aspergillus species remains rare.8 It has been hypothesized that disruption of the complex interaction between amphotericin B and the plasma membrane requires multiple changes in the cell wall, making secondary resistance a challenge for the fungus and hence a rare event.3,8,9

All six patients in this study were severely immunocompromised, with high dose steroids and/or neutropenia playing a prominent role in five of the patients. Besides host factors, pharmacokinetics of amphotericin B may have played a role in the clinical outcome. Christiansen et al.10 proposed that the poor therapeutic activity of amphotericin B in vivo may be because the effective concentration of drug at the site of infection is considerably less than the apparent concentration. They hypothesized that amphotericin B binds tissue components such as cholesterol and lipoproteins in addition to the intended target, ergosterol. The poor outcome seen in four of the patients in this study with cerebral aspergillosis may be explained by subtherapeutic concentrations of amphotericin B in the CNS as a result of both tissue binding and poor penetration.

Our study indicates that use of amphotericin B does not select for resistant mutants during therapy for invasive aspergillosis. In clinical practice, recovery of Aspergillus isolates is infrequent from patients receiving treatment, hence the limited data. These preliminary findings need validation with a larger number of Aspergillus isolates from patients with prolonged exposure to amphotericin B.

Acknowledgements

The authors would like to thank Ms Nafeesa Moosa for her assistance in preparation of this manuscript. Information in this manuscript was presented at the Fortieth Interscience Conference on Antimicrobial Agents and Chemotherapy, September 17–20, 2000, Toronto, Ontario, Canada (abstract no. 1329).

References


Received 27 March 2001; returned 5 July 2001; revised 16 August 2001; accepted 30 August 2001