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In-vitro testing of susceptibility to amphotericin B is a reliable predictor of clinical outcome in invasive aspergillosis

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Invasive aspergillosis is a life-threatening fungal infection which, in neutropenic patients, is associated with an extremely high mortality rate despite optimal treatment. In order to investigate microbiological risk factors for treatment failures in more detail, *Aspergillus* spp. obtained from 29 patients with haematological diseases after myelo-ablative chemotherapy and bone marrow transplantation were analysed for their susceptibility to amphotericin B *in vitro* and this was compared with clinical outcome to see if there was a correlation. *Aspergillus flavus* was present in 12 (41%) of the 29 patients, *Aspergillus terreus* in nine (31%) and *Aspergillus fumigatus* in eight (28%). The susceptibility of these isolates to amphotericin B varied between and within the three species. *A. terreus* was the only organism against which the MIC was consistently high, *A. fumigatus* and *A. flavus* showing variation between isolates in the degree of resistance to amphotericin B. The degree of in-vitro resistance was the only parameter correlating with clinical outcome in a univariate analysis and the only prognostic value in a multivariate analysis considering known risk factors. Irrespective of the species, all six patients with isolates against which the MIC was <2 mg/L survived, whereas most (22/23) of those with isolates resistant to ≥2 mg/L died. Infections among the six survivors were caused by amphotericin B-susceptible *A. fumigatus* and *A. flavus*, but not *A. terreus.* We conclude that the outcome of aspergillus infection depends on the in-vitro susceptibility of the isolates to amphotericin B. Survival was poor in patients with isolates resistant to amphotericin B and good in those with amphotericin B-susceptible specimens. *A. terreus* was always associated with high resistance to amphotericin B and with poor survival.

Introduction

Fungal pathogens are recognized as a major and increasing source of life-threatening infections in the immunocompromised host.[1–4] The increased use of broader-spectrum antibiotics and more aggressive cancer chemotherapy, the increase in transplantation procedures, more aggressive intensive care medicine and the emergence of AIDS are considered the main reasons for this problem.[5] The incidence of fungal infections may vary from 4% in patients with AIDS to 13% in patients undergoing heart transplantation and even 20% in bone marrow transplant recipients.[1,6,7] Data from the National Nosocomial Infections Surveillance System conducted by the Centers for Disease Control have indicated that aspergillosis occurs less frequently than do infections with *Candida* spp. Infections with *Aspergillus* spp. are, however, among the commonest causes of nosocomial pneumonia and are associated with an extremely high mortality rate of 85%.[8] Over the last 30 years, amphotericin B has remained the drug of choice for invasive aspergillosis in immunosuppressed hosts despite its toxicity and low response rates of 30–55%.[1,9,10] Resistance of the fungus to the drug, or inadequate concentration of the drug at the infection site might be responsible for the high mortality rate. In order to investigate in more detail

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the microbiological risk factors for treatment failures, isolates from patients with aspergillus infections were characterized, tested for susceptibility to amphotericin B *in vitro* and the findings were compared with the clinical outcome.

Materials and methods

Patients

All 29 patients with haematological malignancies treated at the Department of Internal Medicine of Innsbruck University Hospital between January 1992 and April 1996 with myelo-ablative chemotherapy and invasive aspergillus infections defined as described previously[1] were considered for this analysis. Diagnosis of invasive aspergillosis included patients diagnosed histologically by biopsy (n = 11) and/or autopsy (n = 20) and those with a characteristic clinical and radiological picture, a culture positive for *Aspergillus* spp. (n = 29) and a lack of response to antibacterial therapy. Underlying diseases were acute lymphocytic leukaemia (n = 4), multiple myeloma (n = 2), lymphoma (n = 1), severe aplastic anaemia (n = 2), Burkitt's lymphoma (n = 2), chronic granulocytic leukaemia (n = 4) and acute myeloblastic leukaemia (n = 14). Detailed clinical characteristics of the patients, including their underlying disease and status, whether there was neutropenia at diagnosis of fungal infection, resolution of neutropenia, surgery and the application of growth factors, are given in Table I.

Treatment with amphotericin B monotherapy was given as prophylaxis in all patients with fever, starting with 0.1 mg/kg after a test dose of 1 mg and escalating to a maximum of 1.5 mg/kg at the time of diagnosis of invasive aspergillosis.

Cultures

For identification of pathogens the clinical specimens (bronchoalveolar lavage, tracheal secretion, sputum and lung biopsy) were first plated on to Sabouraud glucose agar (Merck, Darmstadt, Germany) and incubated at 35°C for 4 days. All fungal strains were identified by their morphology and culture characteristics.

Susceptibility testing

Each isolate was tested for its susceptibility to amphotericin B using a macrodilution technique according to the National Committee for Clinical Laboratory Standards (NCCLS)[11] and to Espinell-Ingroff.[12] The isolates obtained as described above were first cultured on potato dextrose agar slants (Merck) for 7 days at 35°C. Subsequently, a conidial suspension was obtained by flooding each slant with 2 mL of sterile 0.85% saline and the turbidity was measured with a spectrophotometer at 530 nm (DU-64 spectrophotometer; Beckman, Foulerton, MN, USA). Turbidity was adjusted with sterile water to 78–82% for *Aspergillus flavus*, and to 80–82% for *Aspergillus fumigatus* and *Aspergillus terreus.* The adjusted suspensions were diluted thereafter 1 in 100 with culture medium consisting of RPMI 1640 (Whittaker Bioproducts, Inc., Walkersville, MD, USA) buffered with L-glutamine (Sigma, St Louis, MO, USA) and 3-*N*-morpholinopropane sulphonic acid (Sigma) at pH 7.0 to obtain the desired inoculum size of 0.5×10^4–5×10^4 cfu/mL. Of this inoculum 0.9 mL was incubated with 0.1 mL of various drug concentrations of amphotericin B (Squibb, Hounslow, UK) to final concentrations of 16.0–0.03 mg/L. Tubes were incubated at 35°C and observed daily for the presence of growth. The MIC endpoint criterion for moulds was the lowest drug concentration that inhibited ⩾75% of the growth of the tested fungus compared with growth of control. Each result was given as mean ± S.E. from triplicates. As a quality control, testing with *Candida parapsilosis* (American Type Culture Collection, ATCC 22019) was performed as described.

Statistics

The correlation between MICs or factors influencing response to treatment—such as bone marrow transplantation, chemotherapy, neutropenia, disease and disease status, *Aspergillus* spp. and the use of growth factors—and outcome was determined by univariate analysis (Fisher's test) and in a stepwise linear discriminant analysis (multivariate analysis).

Results

Morphology and culture methods showed that *A. flavus* was present in 12 (41%) of the 29 patients, *A. terreus* in nine (31%) and *A. fumigatus* in eight (28%) (Table I). The MICs of amphotericin B against these pathogens were determined as described in Materials and methods. Susceptibility to amphotericin B varied among and within the three species (Table II). The MIC was consistently high (⩾2 mg/L) against *A. terreus* isolates. Some strains of *A. fumigatus* and *A. flavus* were susceptible to <2 mg/L of amphotericin B, while others were resistant to ⩾2 mg/L. The MIC of amphotericin B against the quality control strain *C. parapsilosis* ATCC 22019 was 0.25 mg/L.

Clinical outcome

The lethality from aspergillus infection was 76% (22 out of 29 patients), comparable to that described in the literature.[1] Mortality was dependent on the in-vitro susceptibility of the specimens to amphotericin B. Infections with fungi sensitive to amphotericin B (defined as having a MIC of <2 mg/L) were treated successfully, while 22

Table I. Clinical characteristics and outcome of patients with invasive aspergillosis

Patient no.	Disease	Disease status	Neutropenia[a] at diagnosis of fungal infection?	Neutropenia resolved?	Surgery?	Growth factor	Outcome	*Aspergillus* spp.	MICs (mg/L) of amphotericin B
1	acute myeloblastic leukaemia	first complete remission	no	–	no	–	dead	*A. terreus*	≥2
2	chronic granulocytic leukaemia	chronic phase	no	–	no	–	dead	*A. terreus*	≥2
3	acute myeloblastic leukaemia	first relapse	no	–	no	–	dead	*A. fumigatus*	≥2
4	chronic granulocytic leukaemia	chronic phase	no	–	no	–	dead	*A. terreus*	≥2
5	severe aplastic anaemia	–	no	–	no	–	dead	*A. flavus*	≥2
6	acute myeloblastic leukaemia	first complete remission	no	–	no	–	dead	*A. flavus*	≥2
7	multiple myeloma	progressive disease	yes	yes	yes	–	dead	*A. terreus*	≥2
8	acute lymphocytic leukaemia	refractory	yes	no	no	G-CSF	dead	*A. fumigatus*	≥2
9	chronic granulocytic leukaemia	chronic phase	no	–	no	G-CSF	dead	*A. terreus*	≥2
10	acute myeloblastic leukaemia	second relapse	yes	yes	no	G-CSF	dead	*A. terreus*	≥2
11	acute myeloblastic leukaemia	first relapse	yes	yes	no	G-CSF	dead	*A. flavus*	≥2
12	acute myeloblastic leukaemia	first complete remission	no	–	yes	–	alive	*A. fumigatus*	<2
13	acute myeloblastic leukaemia	first complete remission	no	–	yes	–	alive	*A. flavus*	<2
14	acute myeloblastic leukaemia	first complete remission	no	–	yes	–	alive	*A. flavus*	<2
15	acute myeloblastic leukaemia	first relapse	yes	no	no	G-CSF	dead	*A. fumigatus*	≥2
16	multiple myeloma	first relapse	no	–	no	–	dead	*A. flavus*	≥2
17	acute myeloblastic leukaemia	first complete remission	no	–	no	–	dead	*A. flavus*	≥2
18	acute myeloblastic leukaemia	first relapse	yes	yes	no	G-CSF	alive	*A. fumigatus*	<2
19	acute myeloblastic leukaemia	second relapse	yes	yes	no	G-CSF	alive	*A. fumigatus*	≥2
20	chronic granulocytic leukaemia	chronic phase	yes	yes	no	G-CSF	dead	*A. flavus*	≥2
21	acute myeloblastic leukaemia	first relapse	yes	yes	no	–	dead	*A. terreus*	≥2
22	acute myeloblastic leukaemia	first relapse	yes	no	no	G-CSF	dead	*A. flavus*	≥2
23	acute myeloblastic leukaemia	first chronic phase	no	–	no	–	dead	*A. flavus*	≥2
24	severe aplastic anaemia	–	no	–	no	–	dead	*A. terreus*	≥2
25	acute lymphocytic leukaemia	first relapse	yes	no	yes	G-CSF	dead	*A. terreus*	≥2
26	acute lymphocytic leukaemia	first relapse	yes	yes	no	G-CSF	alive	*A. flavus*	<2
27	Burkitt's lymphoma	progressive disease	no	–	no	G-CSF	dead	*A. fumigatus*	≥2
28	acute myeloblastic leukaemia	first complete remission	no	–	no	–	alive	*A. flavus*	<2
29	Burkitt's lymphoma	first relapse	yes	no	no	G-CSF	dead	*A. fumigatus*	≥2

G-CSF, granulocyte colony-stimulating factor.
[a]Neutropenia defined as absolute neutrophil count $< 0.5 \times 10^9$/L.

of the 23 with resistance to amphotericin B *in vitro* (MIC $\geqslant$ 2 mg/L) died of the fungal infection despite adequate treatment.

All nine patients with *A. terreus* infection died. The lethality of *A. fumigatus* infections was lower than that of *A. terreus* infections: five (63%) of the eight patients died, and all of those who died had a MIC of $\geqslant$2 mg/L. The outcome of patients with *A. flavus* infections (eight (67%) of 12 patients died) was similar to that of patients with *A. fumigatus* infection, again correlating with the susceptibility to amphotericin B. Only patients with susceptible fungi (MIC of amphotericin B, <2 mg/L; $n = 4$) survived, while those with resistant specimens (MIC of amphotericin B, $\geqslant$2 mg/L; $n = 8$) died.

Statistics

Overall outcome correlated significantly ($P < 0.001$, Fisher's test) with MIC values of amphotericin B against *Aspergillus* spp., as shown in Table III. No other variables, such as treatment, stage of disease, presence of neutropenia or the use of growth factors, were associated with outcome ($P > 0.20$, Fisher's test) in a univariate analysis. Using a stepwise linear discriminant analysis, the MIC of amphotericin B against the strain was selected as significantly related to outcome; none of the other tested variables had additional prognostic value.

Discussion

Using high-dose chemotherapy with and without stem cell rescue, considerable progress has been made in treating patients with malignant disease.[13–15] However, as the dose of chemotherapeutic agents given to such patients has increased, the fungal infection rate has increased dramatically, becoming an important determinant for morbidity and, since invasive aspergillosis is associated with extremely high lethality, also for mortality. In the present retrospective analysis we investigated whether isolate-specific characteristics, such as in-vitro susceptibility to amphotericin B, were correlated with clinical outcome. In immunosuppressed patients with similar risk factors such as underlying disease, severity and extent of infection and degree of immunosuppression, we found that the in-vitro susceptibility of the isolates to amphotericin B was the only predictor of outcome. Survival was poor in patients with specimens resistant to amphotericin B *in vitro* and high in those with specimens susceptible to amphotericin B.

Application of in-vitro antifungal susceptibility testing to clinical research and practice was limited until a few years ago as a result of the lack of reproducibility and uncertain clinical relevance.[16] For a variety of reasons standardization of antifungal susceptibility testing is clearly decades behind that for antibacterial susceptibility testing.[17] Until recently, the frequency of fungal infections was a minor problem in the treatment of immunosuppressed patients, the number of available drugs was limited and the potential for resistance to antifungals was not well recognized. During recent years, however, considerable advances have been made with regard to intra- and interlaboratory reproducibility in in-vitro susceptibility testing for yeasts and filamentous fungi.[12] In our hands this method of testing the susceptibility of *Aspergillus* spp. to amphotericin B was reliable, as shown by the low variability in the results of analyses done in triplicate and the reproducibility of the MIC values of the specimens.

With the availability of standardized MIC testing, correlation of the results of susceptibility testing with clinical outcome was found in infections caused by *Candida* spp.[18] Infections with yeasts for which the MIC of amphotericin B was >0.8 mg/L were significantly more often associated with lethality than were infections with *Candida* spp. sensitive to <0.8 mg/L of amphotericin B. Our analysis also reveals such an association for aspergillus infections and might explain the high lethality of

Table II. MIC of amphotericin B against the isolated *Aspergillus* spp.

Species	*n*	MIC (mg/L)
A. terreus	9	$\geqslant$2
A. fumigatus	2	<2
	6	$\geqslant$2
A. flavus	4	<2
	8	$\geqslant$2

Table III. MIC of amphotericin B in relation to survival from disseminated fungal infections

	Outcome of disseminated infection		
MIC (mg/L)	dead	survivors	*P* value (Fisher's test)
<2	0	6	
$\geqslant$2	22	1	<0.001

fungal infection despite adequate treatment. Considering the susceptibility of the isolates in these patients, we might speculate that antifungal drug concentrations were not sufficient to overcome infection. Achieving successful therapy would probably require the maintenance of serum and tissue levels several times higher than the MIC for the offending pathogen.

In our patient population, *A. terreus* strains were consistently resistant to amphotericin B and the predictor for fatal outcome. Our high mortality rate (100%) for *A. terreus* infection is in agreement with reports in the literature showing that disseminated infections with this specimen resulted in death in all patients despite amphotericin B treatment.[19–23] Our in-vitro susceptibility testing suggests that the cause of this poor outcome might be resistance to amphotericin B in this species. The *A. terreus* infection rate observed in our patients (31%) was unusually high since only a few cases of invasive pulmonary disease caused by *A. terreus* have been documented in the literature and *A. fumigatus* and *A. flavus* are the most commonly involved fungi.[24] The reason for this high incidence might reside in the contamination of in-hospital plants with *A. terreus*. While *A. terreus* was constantly resistant to amphotericin B, susceptibility to amphotericin B varied among *A. fumigatus* and *A. flavus*.

Our findings lead us to conclude that susceptibility of the *Aspergillus* spp. is of clinical relevance and varies within and between the species. *Aspergillus* spp. infections in neutropenic patients with amphotericin MICs of $\geqslant$2 mg/L are not treated successfully with amphotericin B. Invasive infections with amphotericin B-resistant specimens might be curable with antifungal combination therapy or specific sensitive antifungal therapy, which might be tested by our in-vitro system. Infections caused by *A. terreus* seem not to be influenced by amphotericin B therapy and are a highly significant predictor of poor prognosis.

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