Epidemiology and management of invasive fungal infections in immunocompromised hosts

Alexander C.A.P. Leenders

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Aan mijn ouders
Voor Marjan en Meilinde

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Chapter 1

General introduction and outline of the thesis.

Alexander C.A.P Leenders

BACKGROUND

Fungal infections in man usually are divided into three categories based upon their major pathophysiological characteristics: superficial and cutaneous, subcutaneous and, systemic infections. The last category consists of two separate entities. First there are the so called "endemic mycoses" caused by dimorphic fungi including *Coccidioides immitis*, *Paracoccidioides brasiliensis, Histoplasma capsulatum* and *Blastomyces dermatitidis* which occur in patients who live in, or have travelled through geographical regions in which these pathogenic fungi have their habitat.[1] Although the consequences of endemic mycoses are sometimes very severe, these infections were not viewed as a very major subject in the field of infectious diseases, probably due to their relatively rare and geographically restricted occurrence. The second entity consists of opportunistic infections caused by fungi that are ubiquitously present around the globe. The number of these infections has increased dramatically during the last three decades. For example, in 1966 invasive aspergillosis was called a disease of medical progress, and world literature on this subject was reviewed in a paper only six pages in length.[2] In contrast in 1990, Denning *et al.* used 55 pages only to review the literature on the treatment of such infections.[3] Several factors have been recognized to be responsible for this rapid increase, all of which are the consequence of advances in medicine. The use of antibacterial agents, the use of cytotoxic chemotherapy, organ transplantation combined with the use of immunosuppressive therapy, the use of indwelling catheters all one way or the other, compromise the defence mechanisms of the human host (Table 1).[4-7] Some of these factors disrupt more than one line of defence; for example, longterm use of steroids, influences cellular immunity, macrophage function and neutrophil function, whereas the integrity of the skin and mucous membranes is little affected.[8]

The number of patients which are immunocompromised due to one or a combination of these factors, has been increasing steadily. In addition, the pandemic caused by the human immunodeficiency virus (HIV) has dramatically further increased the number of patients susceptible to opportunistic fungal infections, especially infections caused by *Candida, Cryptococcus* and *Pneumocystis carinii* (although this last species has only recently been recognized to be a fungus).[9-11] All these developments taken together have pushed opportunistic fungal infections into the category of emerging infectious diseases that urgently require more attention.

Table 1. Factors contributing to the increasing incidence of opportunistic fungal infections.

Immunocompromising factor	Line of defence	Consequence
- Antibacterial therapy	colonization resistance	Overgrowth of *Candida* species
- Indwelling catheter	skin surface	New portal of entry
- Radiation therapy - Cytotoxic chemotherapy	mucosal membranes	New portal of entry
- Cytotoxic chemotherapy - Use of steroids	neutrophils, monocytes, macrophages	Impairment of natural defence against Mucoraceae, *Aspergillus* and *Candida* (invasive disease).
- Use of steroids - Immunosuppressive therapy - HIV	cellular immunity	Impairment of natural defence against *Cryptococcus, Histoplasma, Coccidioides* and *Candida* (superficial and local disease).

Apart from *P. carinii*, three families of fungal pathogens cause the majority of all opportunistic fungal infections: *Candida* spp., *Aspergillus* spp. and *Cryptococcus neoformans*.[12] In addition to these "common" opportunistic infections, a wide variety of other fungal species have shown to be potentially pathogenic to man (Table 2). If man is sufficiently immunocompromised, all fungi seem able to cause infections, making a mycologist call these patients "living Petri-dishes".

SPECIFIC FUNGAL INFECTIONS

Candidiasis

Candida spp. are normal inhabitants of the mucocutaneous surfaces of the body with as main reservoir the gastrointestinal tract and infections are therefore often from endogenous origin.[24] *Candida* spp. most often cause mucocutaneous infections, but under the right circumstances they can also cause hematogenous and deep infections.[24] The spectrum of deep infections ranges from isolated catheter-related candidemia in which signs of dissemination can be minimal to widespread disseminated disease with involvement of multiple organs.

Table 2. Some newly recognized opportunistic fungal pathogens.

Pathogen	Underlying disease/ risk factor	Reference
Phialemonium obovatum	burn wounds	13
Fusarium species	neutropenia	14
Phoma sorghina	chemotherapy	15
Cunninghamella bertolletiae	aplastic anemia	16
Chrysosporium species	acute leukemia	17
Pseudallescheria boydii	liver transplantation	18
Sporotrix schenckii	hairy cell leukemia	19
Trichosporon beigelii	neutropenia	20
Microsporum canis	liver transplantation	21
Paecilomyces lilanicus	neutropenia	22
Xylohypha bantiana	lymphoma	23

At the moment, in the United States (US), the incidence of nosocomial candidemia has quintupled when compared to 1980, and *Candida* spp. is the third most frequently isolated organism in septic patients, directly in line after *Staphylococcus aureus* and *S. epidermidis* but before *Escherichia coli*.[25,26] Most of these infections occur in patients on Intensive Care Units where they frequently receive antibacterial agents and often have multiple indwelling catheters.[27,28] The morbidity and mortality associated with candidemia are impressive. The attributable mortality of this infection has been estimated to range from 21 to 38 % in several studies and the estimated excess length of hospital stay ranged from 14 to 30 days, depending on the underlying illness of patients studied.[29,30] Especially patients given cytotoxic chemotherapy and transplant patients are at risk of developing disseminated candidal infections.[24,30] Since dissemination to multiple organs including kidney, brain, liver, spleen, skin and the eye is common, systemic candidiasis remains a clinical diagnosis for which a high degree of suspicion is necessary. Sensitive diagnostic tests are lacking, blood cultures, for instance, often remain negative.[24] In patients at risk, repeated evaluation for symptoms of dissemination to organs mentioned above should be performed: search for skin lesions, fundoscopy of the eye (in search for characteristic "white cotton-wool" exudates), and documentation of candiduria which can be the first sign of widespread disease. In neutropenic patients the diagnosis is more difficult because a number of symptoms are less likely to occur. It is therefore that only

10-40% of the neutropenic patients have a documented premortem diagnosis of disseminated candidiasis. A presumptive clinical diagnosis based on fever only, often remains, requiring a decision regarding the initiating of antifungal therapy.[31]

This thesis will focus on invasive infections caused by *Aspergillus* and *Cryptococcus* species. However, patients with candidiasis were included in a clinical study of neutropenia-associated fungal infections.

Aspergillosis

Aspergillus spp. are ubiquitously present in dust, soil, water etc. They can cause diseases ranging from allergic aspergillosis to disseminated invasive disease in immunocompromised patients such as hemato-oncologic patients, patients with solid organ transplants and patients on steroids.[32] The number of patients with invasive aspergillosis has been increasing steadily over the past 30 years. In solid organ transplant recipients aspergilli are the most important cause of life threatening invasive fungal infection. The risk of invasive aspergillosis is particularly high in patients with previous cytomegalovirus infection and in those requiring antirejection therapy.[33] Most cases of invasive aspergillosis, however, occur among granulocytopenic patients in which acute invasive pulmonary infection is the most common clinical entity. This reflects the fact that the lungs are the major portal of entry for aspergilli. Infections are often complicated by vascular events including thrombosis and hemorrhage, and by dissemination to the central nervous system, gastrointestinal, skin, bone and cardiac tissues may occur.[32]

The risk of infection in granulocytopenic patients depends largely on exposure to exogenous organisms.[3] The importance of environmental exposure to *Aspergillus* spp. has been reported, for instance when a source was found within a department and in outbreaks during hospital construction activities.[34-36] However, it is not known whether patients become infected with *Aspergillus* strains derived from the hospital, or with strains they already carry with them at admission. Full understanding of the epidemiology of these infections is crucial for the development of preventive strategies. Prevention is particularly important in granulocytopenic patients which are most susceptible to *Aspergillus* infection. The risk of invasive aspergillosis increases with the duration and severity of granulocytopenia.[3,5] In one study the percentage of patients with this infection increased from 0.3% in patients who are granulocytopenic for 17 days, to 3.5% in patients granulocytopenic for 24 days to 14% in patients with a duration of neutropenia of 32 days.[5] Methods that shorten the duration of granulocytopenia

may, thus, be helpful in preventing invasive aspergillosis in these patients.[37,38] Furthermore, it is important to develop protocols describing how the efficacy of preventive strategies can be checked and to study which laboratory techniques can be used for this purpose.

The diagnosis of aspergillosis in the granulocytopenic patient can be difficult to establish due to a lack of inflammatory reactions.[39] Symptoms are often subtle and lesions on chest X-rays can be extremely heterogeneous particularly in the early phases of invasive aspergillosis.[39] More characteristic lesions have been described the last few years, especially on HR-CT of the lungs.[39] Furthermore, the value of respiratory-tract cultures for fungi in the diagnosis of invasive aspergillosis varies largely between the various groups of patients, the overall sensitivity being disappointing, while positive predictive values in patients with granulocytopenia is generally high.[40] Bloodcultures are seldomly positive and don not add to the diagnosis of invasive aspergillosis. The lack of signs and symptoms and sensitive diagnostic tests must lead to a high level of suspicion in febrile granulocytopenic patients not responding to broad-spectrum antibacterial therapy, especially when dyspnea, cough or pleuritic pain is present. Clearly, better non-invasive diagnostic tests (such as PCR, ELISA) are needed, even more so because early diagnosis may improve outcome.[41,42]

Cryptococcosis

Cryptococci are found all over the world, particularly in association with bird droppings, but they also may be isolated from soil and decaying vegetables and fruits.[43] Because the respiratory tract is the portal of entry, pulmonary cryptococcosis is probably the most common manifestation of cryptococcal infection.[43] However, because this initial infection usually goes unnoticed, cryptococcal meningitis is by far the most reported clinical manifestation and by definition is considered to be a disseminated infection.

Prior to the HIV pandemic, *C. neoformans* infections were reported only sporadically (estimated to be one or two new cases per 1 million inhabitants) in the US.[44,45] This number rapidly increased and cryptococcosis became the fourth most common opportunistic infection among patients with AIDS, with a frequency estimated to be between 1.9 and 11.6% in retrospective studies.[46,47] The number of AIDS patients with cryptococcosis varies greatly by geographic area reaching almost 30% among patients in certain areas in Western Africa.[48] Epidemiologic studies must show if this is due to the presence of more virulent strains of *C. neoformans* in Africa or merely to increased exposure to this fungus. The last few

years the incidence of infections caused by *C. neoformans* seems to decline, possibly due to the extensive use of azole therapy for candidiasis in these patients. The recent introduction of highly active anti-retroviral therapy (HAART) may well lead to a further decline in the incidence of opportunistic infections and so to infections caused by *C. neoformans*.

Cryptococcal meningitis in HIV-infected patients is an indolent disease, in which symptoms, usually headache, fever and malaise, may be present for weeks. Neck stiffness and focal neurologic signs are relatively uncommon.[49] Prostatitis occurs frequently, and the prostate may serve as a focus for relapsing infection. Mortality from cryptococcal meningitis in HIV-infected patients ranges up to 22%.[50] Prognostic factors for outcome in non-HIV-infected patients can not automatically be applied to HIV-infected patients and should be studied in this group (chapter 8).

THERAPY FOR INVASIVE OPPORTUNISTIC FUNGAL INFECTIONS.

Because fungal infections were for a long time not considered to be a major issue in clinical medicine, the limited range of effective antifungal agents has not caused much concern. Even today, only three classes of drugs are available for the treatment of systemic fungal infections: the polyenes, flucytosine and the azoles.

Polyenes

Amphotericin B (Amb) belongs to the polyenes which were the first drugs available that possessed antifungal activity. Until 30 years ago, when a patient had an invasive fungal infection, the only option for treatment was Amb. Today, it is still the agent of choice for the treatment of most invasive fungal infections.[51-53] Of all known polyenes it is the only formulation that has antifungal activity and that can be administered intravenously. The antibiotic is produced by *Streptomyces nodosus* and acquired its name from the fact that the drug is amphoteric. Amb is available as a commercial preparation for parenteral use (Fungizone), which also contains desoxycholate and sodium phosphate buffer. Amb binds to ergosterol within the fungal cell wall and, thus, alters the membrane permeability, causing leakage of cellular components and eventually cell death.[53,54] Furthermore Amb can damage the fungal cell wall by the formation of free oxygen radicals.[54]

Amb has good activity against most clinically important fungi, with the exception of *Pseudallescheria boydii, Fusarium spp, Candida lusitaniae* and

dematiaceous fungi. A major drawback of this agent is its toxicity, which is postulated to be caused by its affinity for cholesterol in the membranes of mammalian cells.[55] Renal toxicity is clinically the most significant potential adverse reaction. Glomerular filtration rate, renal blood flow and tubular reabsorption may all decrease leading to tubular acidosis, azotemia, oliguria and loss of magnesium and potassium.[56-58] Mostly these effects occur early during treatment (<2 weeks) and are reversible, however, irreversible renal damage has been reported albeit rarely.[56,58,59] Other side effects are fever and chills during infusion, anemia, thrombophlebitis (making infusion through central catheters the preferred route), nausea, vomiting and several others.

Although it is recommended to use Amb in as high a dosage as possible, the adverse effects usually limit the dose that can be tolerated to 0.7-1.5 mg/kg/-day.[3,51,60] These relatively low dosages may explain, in part, the treatment failures observed with Amb use. The proportion of patients which fail on therapy is particularly high in immunocompromised patients treated for mould infections.[3] Because the major limiting factor for the use of Amb is its toxicity, a number of strategies have been explored to overcome this problem. One of these strategies is the use of lipid-based formulations of Amb (see chapter 5). This same strategy was used in an attempt to make the intravenous administration of nystatin (another fungicidal polyene) possible.[61]

Flucytosine

Flucytosine (5FC) is a fluorine analogue of cytosine which has antifungal activity after its conversion to the antimetabolite 5-fluorouracil.[62] Its activity consists of both inhibition of RNA synthesis and interruption of DNA synthesis.[63]

5FC is most effective in the treatment of yeast infections, although it also has activity against moulds. *H. capsulatum, C. immitis, B. dermatitides* and *P. boydii*, however, are usually resistant.[62] Besides minor gastrointestinal complaints, 5FC monotherapy rarely gives rise to side effects.[62] Unfortunately, when 5FC is used alone, resistance develops rapidly, probably by selecting resistant variants. Thus, 5FC is usually given in combination with Amb. This combination frequently results in raised serum levels of 5FC caused by Amb induced renal impairment.[64] Serum levels of 5FC >100 μg/mL may induce bone marrow depression resulting in neutropenia, thrombocytopenia or pancytopenia.[65,66] If 5FC is used in combination with Amb, it is, therefore, recommended that serum levels of 5FC be monitored twice weekly.[67]

At this point in time cryptococcal meningitis remains the only infection in

which addition of 5FC to Amb has proven to improve treatment outcomes.[68,69] Furthermore, 5FC can be added in cases of serious candidal infections if the response to Amb alone is not satisfactory. The usefulness of 5FC in cases of invasive aspergillosis is still controversial due the lack of data on it's clinical efficacy.[53]

Azoles

In this era of increased interest in invasive fungal infections, new agents within the class of the azoles are being developed.[70] Most important is the development of the tri-azoles itraconazole and fluconazole, by *N*-substitution of the imidazoles.[71] The affinity of these tri-azoles for the human sterol synthesis is remarkably lower than for the fungal sterol synthesis. Side effects seen with the use of, the non-N-substituted ketoconazole (hormonal suppression and many drug interactions) are greatly diminished.

Azoles act by inhibiting 14-alfa-demethylation of fungal lanosterol by binding to cytochrome P-450. This leads to reduced concentrations of ergosterol, a sterol essential for the fungal membrane, and subsequently to a more permeable cytoplasmic membrane. This results in leakage of intracellular components. Azoles are mainly fungistatic, although fungicidal activity, probably due to direct membrane damage has been demonstrated at higher concentrations.[72]

Adverse effects are uncommon with the use of the tri-azoles, and consist mainly of gastro-intestinal complaints. The use of itraconazole and fluconazole may be complicated to a lesser extent by hepatotoxicity, a major problem with the use of ketoconazole.[70]

Fluconazole is active mainly against the yeasts *Candida* (but not *C. krusei* and *C. glabrata*) and *Cryptococcus.* Coccidioidomycosis and sporotrichosis however, can also be treated with fluconazole, but the drug appears to be ineffective in aspergillosis.[73,74] Fluconazole is used in mucosal, cutaneous and invasive candidiasis and as follow-on and maintenance therapy in cryptococcosis.

Itraconazole has activity against a broad spectrum of fungi including *Blastomyces, Histoplasma, Aspergillus, Candida* and *Cryptococcus.*[75] Unfortunately, up to now, itraconazole is only available in an oral formulation. Particularly in granulocytopenic patients, this formulation results in unpredictable serum levels, and therapy failures in patients with low serum levels have been described. A recently marketed liquid oral formulation may overcome this problem, but serum levels should be monitored, certainly in the immunocompromised patient.

Antifungal therapy for specified infections.

Due to differences in the spectrum of antifungal agents, different regimes are employed in the treatment of different infections. In patients with invasive candidiasis caused by *Candida* spp. other than *C. krusei* or *C. glabrata*, fluconazole is the agent of choice.[76,77] However, when these patients are granulocytopenic, many clinicians will start with a course of AmB and switch to fluconazole only when the number of neutrophils has normalized. Infections with *C. krusei* or *C. glabrata* need to be treated with AmB. With the available agents, mortality remains high, and other regimes remain warranted.

Optimal treatment for cryptococcosis in non-HIV-infected patients consists of AmB with 5FC followed by fluconazole.[68,78] It was not clear whether this would also be the preferred regime in HIV-infected patients.[79,80] Concern existed about the toxicity of 5FC in these patients especially the potential bone-marrow suppressive effects. Clearly there is a need for less toxic regimes with at least comparable efficacy. The principal goal of the treatment of HIV-associated cryptococcal meningitis remains to gain control over the acute illness and then to continue with lifelong maintenance therapy to prevent relapses.

Invasive aspergillosis is usually treated with AmB in doses as high as patients can tolerate.[3] Despite these high doses, treatment fails in a high percentage of patients. Itraconazole is an alternative to AmB, but response rates remain <50%.[81] Problems with the resorption are a major drawback in the use of itraconazole in granulocytopenic patients and it is often only used as follow-on therapy when patients have recovered from neutropenia. More active and less toxic agents particularly for immunocompromised patients are necessary.

OUTLINE OF THE THESIS

At this moment the number of drugs that are effective in the treatment of the increasing number of opportunistic fungal infections remains very limited. The most widely used drug (Amb) has major side effects, and failure rates remain high. This underscores the importance of preventing these infections. Elucidation of the epidemiology of opportunistic fungal infections is a prerequisite for the development of effective preventive strategies. For example, do patients become infected with *Aspergillus* strains derived from the hospital, or with strains they already carry with them at admission, how do preventive strategies influence the density of conidia and how can one study the impact of these strategies? Furthermore, there is a clear need for more effective, less toxic antifungals and for this purpose new antifungal drugs have been developed and studied for their efficacy. One of the promising developments are the lipid-based products of AmB, which may have an increased therapeutic index. This, however, should be proven in randomized studies for each of the individual products.

This thesis is called "Epidemiology and management of invasive fungal infections in immunocompromised hosts", and particularly focusses on two opportunistic fungal infections in two distinct groups of hosts: pulmonary aspergillosis in granulocytopenic hosts and cryptococcal meningitis in HIV-infected patients. The aim of the study was two-fold:

1. to evaluate the role of randomly amplified polymorphic DNA (RAPD) in the elucidation of the (aero)-epidemiology of aspergillus infections and the epidemiology of cryptococcosis and to answer some of the epidemiological questions.
2. to study the toxicity and efficacy of one of the lipid-based formulations of amphotericin B (AmBisome) in the treatment of pulmonary aspergillosis and cryptococcal meningitis.

In **chapter 2** we describe how we used RAPD to try to elucidate the cause of an apparent outbreak of invasive aspergillosis at the hematology ward of the University Hospital Rotterdam-Dijkzigt. After this outbreak we started a surveillance of fungal conidia in the hospital air and in the air outside. Results of this surveillance and further studies on the obtained isolates using RAPD, are described in **chapter 3.**

Within a major typing study, we used RAPD to study the genetic relatedness of clinical isolates of *C. neoformans* and compared isolates of *C. neofor-*

mans varii neoformans with *C. neoformans varii gatii* (**chapter 4**).

In **chapter 5** a general review is given of the use of lipid based formulations of amphotericin B in animal models and in clinical settings. To study the efficacy of one such formulation (AmBisome) we developed a rat-model of one-sided pulmonary aspergillosis (**chapter 6**). This same formulation was compared to "conventional amphotericin B" (Fungizone) for toxicity and efficacy in the treatment of invasive fungal infections in granulocytopenic patients. Results of this study in which mainly patients with pulmonary aspergillosis were entered are described in **chapter 7**.

In **chapter 8** we retrospectively looked for prognostic factors for the outcome of HIV-infected patients with cryptococcal meningitis. These factors were used in the evaluation of the results of a comparative trial of AmBisome and Fungizone in which HIV-infected patients from centers in the Netherlands and Australia with this infection were studied (**chapter 9**).

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Chapter 2

Molecular epidemiology of apparent outbreak of invasive aspergillosis in a hematology ward.

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ABSTRACT

During a two month period, five patients suffering from invasive infections caused by *Aspergillus flavus* or *Aspergillus fumigatus* were identified in the Hematology Department of the University Hospital Dijkzigt (Rotterdam, the Netherlands). To study the epidemiological aspects of invasive aspergillosis, strains from these patients and from the hospital environment, isolated during extensive microbiological screening, were subjected to genotyping. A novel DNA-extraction technique, involving freezing, grinding and direct lysis in guanidium isothiocyanate containing buffers of mycelial material, was applied. DNA isolation was followed by typing by random amplification of polymorphic DNA (RAPD) analysis. This showed that strains isolated from all patients infected with the same fungal species were genotypically distinct thus providing evidence against the possibility of an ongoing, single source nosocomial outbreak. Strains could also be differentiated from strains of geographically diverse origins. However, one of the patients' strains of *A. flavus* was also frequently encountered in the hospital environment. As all environmental strains were collected after this patient had been diagnosed with invasive disease, the epidemiological value of this observation could not be ascertained. Intensive investigations showed no single source of *A. flavus* or other aspergilli. RAPD genotyping proved that the outbreak of invasive aspergillosis in the hematology ward consisted of a series of unrelated events and was not due to a common source within the hospital. RAPD fingerprinting of aspergilli may greatly facilitate future investigations on the epidemiology of invasive disease with these pathogens.

INTRODUCTION

Aspergillus spp. are widely distributed fungi which, after inhalation of their conidia, can cause invasive, usually pulmonary infection in immunocompromised patients.[1] In oncology patients with prolonged duration of neutropenia, *Aspergillus* spp. infections represent an increasing problem.[2] *A. fumigatus* is by far the predominant species in cases of invasive disease; *A. flavus* is isolated less frequently but more often causes extrapulmonary invasive infections.[3,4] As the prophylactic and therapeutic efficacy of the present antifungal agents is insufficient in many cases, it is important to protect susceptible patients from inhaling *Aspergillus* conidia. Adequate protective isolation using High Efficiency Particulate Air (HEPA) filtration currently provides the best barrier against invasive fungal disease. Nevertheless, small outbreaks of nosocomial aspergillosis have been reported, sometimes in association with environmental sources of conidia.[5,6] Elucidation of the complex epidemiology in such cases requires detailed molecular typing studies. However, not all of the molecular procedures are applicable to fungal typing.[5,7-9]

During an apparent outbreak of nosocomial aspergillosis in the University Hospital Rotterdam, clinical and environmental isolates of *A. flavus* and *A. fumigatus* were collected. The possible role of *Aspergillus* genotyping in managing such outbreaks was investigated, and measures taken at the time of the outbreak were retrospectively re-evaluated. The present report describes a novel DNA extraction method and the application of randomly amplified polymorphic DNA (RAPD) analysis as genotyping method. The value of different primers for distinguishing between strains of *A. flavus* as well as *A. fumigatus* was determined.

MATERIALS AND METHODS

This section describes, under two separate headings, the epidemiological and patient-related procedures and the laboratory techniques for typing of isolates of *Aspergillus* spp.

Patients and environmental screening.

Invasive aspergillosis. A patient was diagnosed to have invasive aspergillosis when *Aspergillus* spp. was cultured, in the absence of other pathogens which

could explaining clinical symptoms, from broncho-alveolary lavage (BAL) fluid or from deep tissue specimens. Invasive disease was confirmed by histopathology. X-ray analysis and drug response were considered additional arguments corroborating invasive disease. Five patients were diagnosed with invasive aspergillosis within the hematology ward in the period from May to July 1994 (Fig. 1).

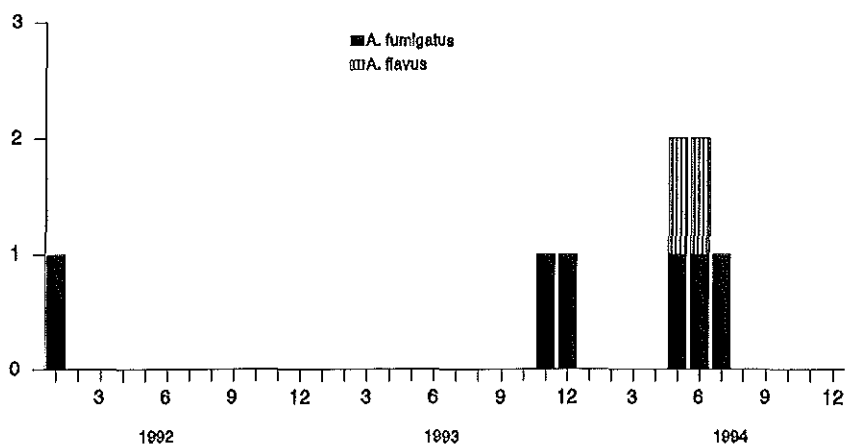

Figure 1. *Number of patients diagnosed with invasive aspergillosis during 3 consecutive years. The numbers 1 to 12 on the x-axis correspond to the months January to December.*

Prophylaxis. To prevent development of invasive aspergillosis in other patients, prophylactic measures were taken. Windows were kept closed in the entire hospital. Patients in the hematology ward received one of two antifungal prophylactic regimens on a daily basis: 10 mg aerosolized amphotericin B in combination with either itraconazol (200 mg orally) or amphotericin B (0.5 mg/kg intravenously). This last regime was given to patients who were neutropenic (neutrophilic granulocytes $< 0.5x10^9$) during the outbreak period, because of potential exposure to high levels of fungal conidia.

Air sampling. To study the numbers of conidia in the hospital air, serial air samples of 1 cubic meter each, were taken twice a week with an Surface Air System (SAS)-sampler containing Sabouraud agar plates, which were incubated at 22°C and 37°C to examine growth of both non-pathogenic and pathogenic fungi. At the same time similar air samples were taken just outside the hospital and at a nearby demolition site.

Air-conditioning and filtration systems. To investigate the air-conditioning systems and HEPA filters, patients were temporarily moved to a new building. During the first two weeks of closure of the old department several air samples were taken. After this period, the ceilings were removed and analyzed for fungal growth. The air-conditioning and filtration systems were opened and also studied. Swabs and air samples were taken from within the systems and other suspicious sites. HEPA filters were removed, studied for fungal growth and new filters were replaced.

Fungal strains and DNA typing.

Fungal isolates. Isolates of *A. flavus* and *A. fumigatus* were collected from patients with invasive aspergillosis nursed in the Hematology ward of the University Hospital Dijkzigt (Rotterdam, the Netherlands). Additional isolates were collected from patients treated on other wards and from various environmental sources (within and outside the hospital). Additional clinical isolates of *A. flavus* were obtained from the Department of Medical Microbiology, University Hospital Nijmegen (the Netherlands), the Regional Laboratory for Public Health Haarlem (the Netherlands) and the Diagnostic Microbiology Laboratory of the University of Minnesota (Minneapolis, USA). Species were identified on the basis of culture characteristics and morphology of conidiophores and conidia. Strains were propagated on Sabouraud media and stored at -70°C in Brain Heart Infusion (BHI) broth containing 10% glycerol.

Fungal DNA isolation. Strains were inoculated in 25 ml Sabouraud maltose medium, containing 4 mg/kg gentamicin, and incubated at 37°C for 72 h until abundant mycelial growth was observed. The entire thallus was collected in a porcelain bowl. Liquid nitrogen was added, after which grinding of the frozen material took place. While working in a safety cabinet, between 10-25 ml lysis buffer (0.1 M Tris-HCl pH 6.4, 40 mM EDTA pH 8.0, 1% Triton X-100, 4 M guanidium isothiocyanate) was added and this suspension could be stored for variable periods at -20°C. DNA degradation was prohibited by the immediate lysis

of destructed cells and guanidium isothiocyanate induced protein denaturation. One ml of the extract was centrifuged for 5 minutes (15,000 rpm). To the supernatant 100 µl of a Celite suspension (200 mg/ml) (Aoroa organics, Grel, Belgium) was added and this suspension was shaken vigorously by hand. The pellet was washed in a second lysis buffer (0.1 M Tris-HCl pH 6.4, 4M guanidium isothiocyanate), ethanol 70% and acetone, respectively.[10] After drying, the pellet was resuspended in 150 µl of 10 mM Tris-HCl pH 8.0 and incubated at 56°C for 10 minutes. Approximately 125 µl of the supernatant was collected. The DNA concentration was estimated by electrophoresis of DNA-containing aliquots through 1% agarose-gels, run in 0.5xTBE in the presence of ethidium bromide, and comparison with the staining intensities of known amounts of bacteriophage lambda DNA.

PCR-mediated DNA fingerprinting. DNA typing by RAPD was performed as described previously.[11-13] Approximately 50 ng of fungal DNA was subjected to 40 cycles of repeated denaturation (1 min 94°C), primer annealing (1 min 25°C) and enzymatic chain extension (2 min 74°C) in 100 µl of a buffer-system consisting of 10 mM Tris-HCl pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton-X100, 0.2 mM dNTPs, 50 pmol of primer and 0.5 units of *Thermus thermophilus* DNA polymerase (Sphaero Q, Leiden, The Netherlands). Initially, the enterobacterial repetitive intergenic consensus primers ERIC-1 and ERIC-2 were employed in separate assays (Table 1 and reference 6). Sixteen additional primers were evaluated with respect to their resolving power towards *Aspergillus* spp. DNA. The additional primers selected on the basis of these experiments are listed in Table 1. The resulting banding patterns were indexed by capital lettering and even a single band difference led to a different letter code. Differences found in ethidium bromide staining intensities were ignored. Upon combination of the results of multiple assays a single, 7-letter genocode was deduced. Banding patterns were interpreted visually by three independent persons. Reproducibility and reliability were assessed as suggested previously.[5]

PCR ribotyping. PCR ribotyping was performed as described for a number of bacterial pathogens.[14,15] Employing primers SP1 and SP2 (Table 5) the 16S-23S intergenic spacer region was amplified using a PCR program consisting of forty cycles of alternating denaturation (1'94°C), primer annealing (1'55°C) and primer extension (1'74°C). The reaction components were identical to those described above.

All PCRs were performed in a Biomed type 60 thermocycler (Biomed,

Theres, Germany) and, after amplification, the DNA fragments were length-separated by electrophoresis through 0.8-2% agarose gels in 40 mM Tris.Acetate pH 7.4, 1 mM EDTA, 0,33 μg/ml ethidium bromide. Results were documented by Polaroid photography using a Land camera and Polapan 556 High Speed films.

Table 1. *DNA-sequences of primers used.*

Primer[a]	Code	Sequence (5' - 3')	Reference
E1	ERIC-1	CAC TTA GGG GTC CTC GAA TGT A	6
E2	ERIC-2	AAG TAA GTG ACT GGG GTG AGC G	6
3	RAPD-1247	AAG AGC CCG T	this paper
4	RAPD-1254	CCG CAG CCA A	this paper
5	RAPD-1281	AAC GCG CAA C	this paper
6	RAPD-1283	GCG ATC CCC A	this paper
26	BG-2	TAC ATT CGA GGA CCC CTA AGT G	this paper
28	REP 2-I	ICG ICT TAT CIG GCC TAC	6
sp1	Spacer-1	TTG TAC ACA CCG CCC GTC A	25
sp2	Spacer-2	GGT ACC TTA GAT GTT TCA GTT C	25

[a]Primers 3 and 4 and primers 5 and 6 were used in combination in one assay.

RESULTS

Patients and environmental screening.

Outbreak. Within a period of 45 days during the early summer of 1994, 5 patients from the hematology ward were diagnosed with invasive aspergillosis (Fig. 1). During the preceding 24 months only two patients with invasive aspergillosis had been diagnosed in the same ward. This indicated a sudden and significant increase in infection incidence. *Aspergillus* spp. was cultured from BAL fluid from two patients, without the simultaneous culturing of other pathogens. Both patients had chest X-rays concomitant with invasive aspergillosis. *Aspergillus* spp. was also cultured from biopsy material from the eye, from the mastoid air cells and from the sinus sphenoidalis of an additional three patients (Table 2). No other micro-organisms were cultured from these biopsy materials. Calco Fluor White

Table 2. Patients with invasive disease caused by A. flavus or A. fumigatus.

Patient		*Aspergillus* infection			
gender[a] age	Hematological disease	Date of diagnosis	clinical manifestation	pathogen	Outcome
F;38	Multiple myeloma	27-05-94	Endophthalmitis	*A. fumigatus*	Alive
M;37	Acute myeloid leukemia	01-06-94	Invasive external otitis	*A. flavus*	Alive
M;53	Acute lymphocytic leukemia	30-06-94	Pneumonia	*A. fumigatus*	Alive
M;65	Aplastic anemia	02-07-94	Sinusitis	*A. flavus*	Died
M;40	Chronic myeloid leukemia	10-07-94	Pneumonia	*A. fumigatus*	Died

[a]F = female, M = male.

stainings clearly showed the true invasiveness of the fungi. After prophylactic regimens were started, no additional patients with invasive aspergillosis were diagnosed.

Air samples. High numbers of aspergilli were cultured from air samples taken from the outside air and, albeit at lower densities, from air samples taken within the hospital (Table 3). Only on two occasions did the number of aspergilli cultured on the hematology ward exceed the numbers cultured from other departments. On both occasions this was due to *A. flavus*. Two HEPA-filtered rooms also yielded significant growth of *A. flavus* on these occasions. Air samples taken at a nearby demolition site showed heavy growth of aspergilli (*A.fumigatus*, *A. flavus* and *A. niger*). However, the number of conidia sharply decreased if one moved away from this site.

Table 3. *The number of CFU's[a] of all fungi (pathogenic and non-pathogenic) in the hospital air and the surroundings, during subsequent study periods. The numbers in parentheses refer to Aspergillus spp. only.*

Period[b]	Outside		Hematology ward		HEPA-filtered rooms	
I	530	(0.9)	9.1	(0.04)	2.0	(0.02)
II	340	(1.3)	2.4	(0.02)	0.3	(0)
III	200	(1.1)	0.8	(0)	0.5	(0.03)

[a]Numbers shown are the average CFU's/m^3 deduced from totals of 15m^3 (outside), 20m^3 (HEPA) or 50m^3 (hematology ward).
[b]Period I is the outbreak period, period II the two weeks in which the ward was closed and III is the period in which the air-conditioning systems were opened and examined.

Air conditioning and filtration systems. In the first two weeks after the old department was closed, the number of aspergilli cultured, sharply decreased. Air samples from above the ceiling-plates, taken after this period, showed no growth of fungi, nor did swabs taken from surfaces that looked suspect. All samples taken from within the air-conditioning and filtration systems failed to grow aspergilli. Also swabs taken from HEPA filters and pieces of these filters placed on Sabouraud plates revealed no fungal growth. Fungal isolates (all *A. fumigatus*)

Table 4. PCR typing of nosocomial and environmental A. flavus isolates.

Isolate				Genotype using PCR primer species							Overall RAPD
no.	code	Source[a]	Isolation date	26	28	3/4	5/6	E1	E2	sp1/2	type[b]
1[c]	2-9	Sinusitis	02-07-94	A	A	A	A	A	A	A	I
2[c]	1-76	Inv. otitis ext.	01-06-94	B	B	B	B	B	B	B	II
3	6-3	Sputum elsewhere	22-07-94	C	A	C	C	A	A	A	III
4	2-45	HEPA hematology	07-07-94	A	A	A	A	A	A	A	I
5	4-62	Hematology ward	19-07-94	A	A	A	A	A	A	A	I
6	2-15	Hematology ward	04-07-94	D	C	D	D	C	B	B	IV
7	2-36	Internal ward	06-07-94	A	A	A	A	A	A	A	I
8	6-46	Environment	05-08-94	E	D	E	E	A	A	A	V
9	4-61	Hematology ward	19-07-94	A	A	A	A	A	A	A	I
10	4-70	Hematology ward	19-07-94	A	A	A	A	A	A	A	I
11	5-73	Environment	26-07-94	F	C	F	F	D	C	A	VI
12	5-33	Environment	18-07-94	G	E	G	G	A	C	B	VII
13	143	Superficial isolate RLH	NA[d]	H	F	nd[e]	H	E	D	A	VIII
14	2062	Superficial isolate RLH	NA	I	C	A	I	F	A	A	IX
15	884	Superficial isolate RLH	NA	J	G	A	J	A	E	A	X
16	1826	Superficial isolate RLH	NA	I	H	H	K	F	F	B	XI
17	1220	Superficial isolate RLH	NA	K	I	I	L	G	G	B	XII
18	985	Superficial isolate RLH	NA	J	G	A	J	A	E	A	X
19	137	Superficial isolate AHN	NA	C	J	C	C	A	A	A	XIII

20	284	Bronchial isolate AHN	NA	L	J	K	N	F	I	B	XIV
21	509	BAL isolate AHN	NA	M	J	K	N	F	I	B	XV
22	510	Obduction isolate AHN	NA	N	K	L	O	H	J	B	XVI
23	511	Obduction isolate AHN	NA	N	K	L	O	H	J	B	XVI
24	512	Obduction isolate AHN	NA	N	K	L	O	H	J	B	XVI
25	513	Obduction Isolate AHN	NA	N	K	L	O	H	J	B	XVI
26	544	Abdominal isolate AHN	NA	O	J	M	P	I	K	A	XVII
27	744	Superficial isolate AHN	NA	C	L	C	C	J	L	A	XVIII
28	1023	Superficial isolate AHN	NA	J	J	N	J	K	M	A	XIX
29	432619	Clinical isolate USA	NA	P	M	O	Q	A	C	A	XX
30	433771	Clinical isolate USA	NA	Q	N	P	R	L	N	A	XXI
31	506857	Clinical isolate USA	NA	R	O	Q	S	M	O	C	XXII
no.types				18	15	17	19	13	15	3	22

[a]RLH = Regional Laboratory Haarlem, AHN = Academic Hospital Nijmegen, USA = Diagnostic Microbiology Laboratory of the University of Minnesota (Minneapolis).

[b]The overall PCR type was based on the number of differing banding patterns when all assays were combined.

[c]Clinical isolate of patient listed in Table 2.

[d]NA = not available.

[e]nd = not done.

were obtained from the inlet-filters of the HEPA-filtration systems.

Fungal strains and DNA typing.

Fungal isolates. Two isolates of *A. flavus* were collected from patients with invasive disease, hospitalized in the hematology ward. An additional strain was obtained from the sputum of a patient on another ward. Twenty five isolates were obtained from air-samples taken during the study period. Nineteen additional strains were obtained from other institutions (Table 4).

Three isolates of *A. fumigatus* from patients with invasive infection in the hematology ward and four clinical isolates from patients on other wards were collected. From the environment, eight strains isolated during the study period were chosen randomly (Table 5).

Fungal DNA isolation. The protocol combining grinding and immediate lysis in guanidium isothiocyanate containing buffers, enabled high efficiency, high quality DNA isolation. DNA preparations thus obtained, allowed reproducible RAPD analysis. This was tested by using different batches of DNA isolated on different days from different fungal strains (data not shown).

Comparing isolates of A. flavus. Full scale RAPD analysis was first performed on a selection of the strains from Rotterdam (n=12) (Table 4, Fig. 1). Several different types were discovered: out of twelve isolates, six appeared to be unique, whereas one pattern was found six times. Analysis of 16 additional strains from Rotterdam, with three RAPD primers only, revealed genetic heterogeneity in this group; seven different strains could be distinguished (data not shown). The pattern that was common among the first group, was again found nine times. The other strains appeared to be unique. Overall, 15 of the 28 nosocomial isolates were identical or highly similar; one of these was a strain recovered from one of the patients with invasive disease (patient 4). The combined RAPD pattern of each of the two other clinical isolates was unique.

The *A. flavus* isolates from within the Academic Hospital Rotterdam could be differentiated from those obtained from the other institutions. Moreover, these latter isolates could be differentiated from each other, although identical RAPD results were sometimes found within geographically clustered groups (for instance H3 and H6, see Figure 2). Documentation on possible epidemiological relationship between these strains was not available. Among the strains from the University Hospital Nijmegen, five had been derived from a single deceased patient. Interestingly, post-mortem strains cultured from different organs of this

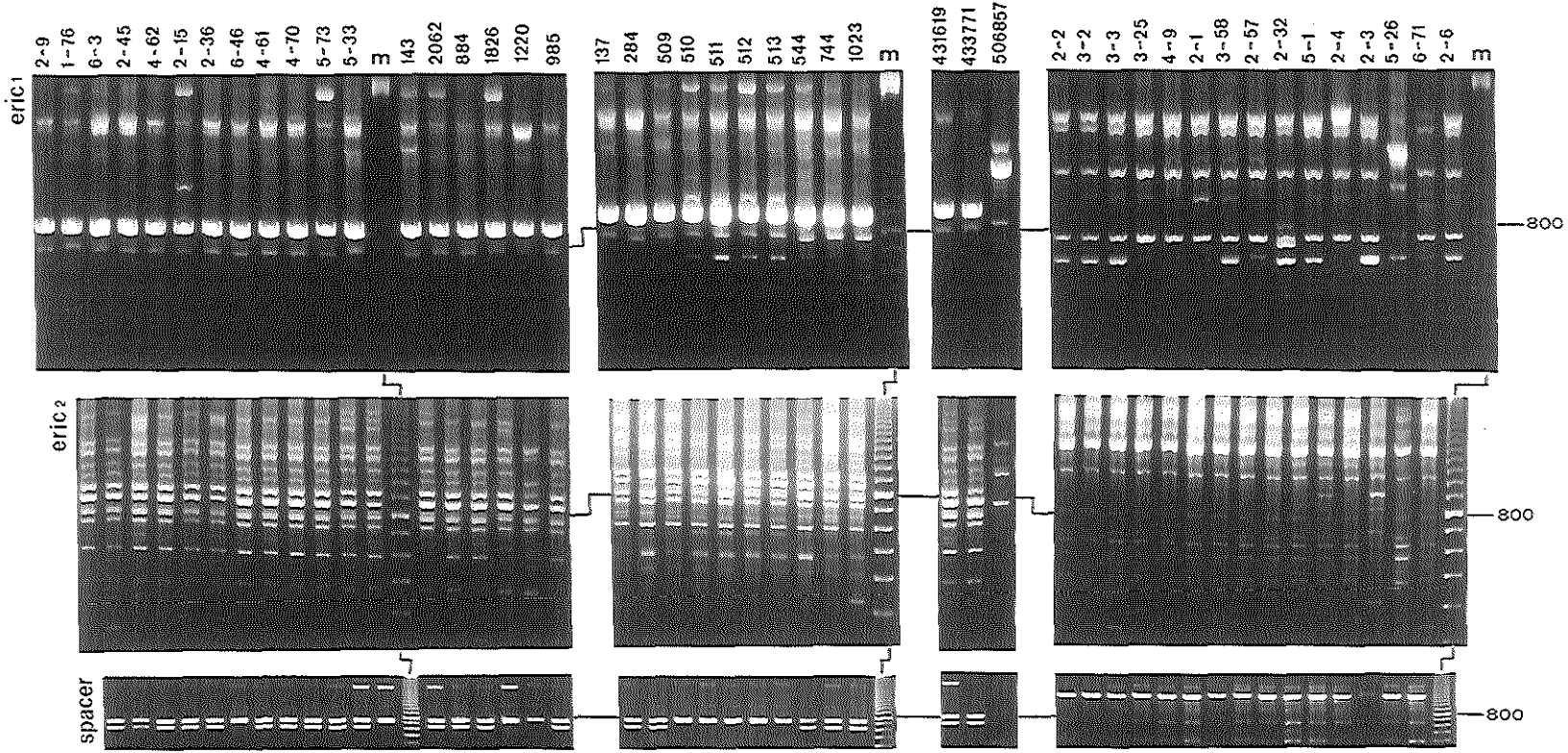

Figure 2. *PCR typing of nosocomial and environmental isolates of A. flavus and A. fumigatus strains. From left to right, first the A. flavus strains are assayed. The numbers given above the lanes correspond with the codes given in Table 4. In the right hand part of the figure, results obtained with the A. fumigatus strains are displayed. The numbering above the lines (2-2 through 2-6) corresponds with the codes given in Table 5. Lanes marked "m" contain the molecular length marker, of which the prominent 800 basepairs fragment is highlighted on the right. The top panel shows results of AP PCR performed with ERIC-1 primer, the middle panel shows the ERIC-2 data. The bottom panel illustrates the ribotyping as performed for all fungal strains. For these tests the sp1/sp2 primerpair was employed. The interpretation of the pictures is given in Tables 4 and 5.*

Table 5. PCR typing of nosocomial and environmental A. fumigatus isolates.

Isolate				genotype using PCR primer species							Overall RAPD
No.	code	Source[b]	Isolation date	26	28	3/4	5/6	E1	E2	sp1/2	type[a]
1	2-2	CF-patient elsewhere	01-06-94	A	A	A	A	A	A	A	I
2	3-2	Hematology ward	09-07-94	A	A	B	B	B	B	A	II
3	3-3	Hematology ward	09-07-94	A	A	B	B	B	B	A	II
4	3-25	Environment	13-07-94	B	B	C	C	C	C	A	III
5	4-9	Environment	11-07-94	A	C	D	D	D	D	A	IV
6	2-1	BAL ptt. elsewhere	04-07-94	C	C	D'	E	E	E	A	V
7	3-58	Hematology ward	12-07-94	C	A	A	F	B	B	A	VI
8	2-57	Environment	08-07-94	A	C	A	G	F	A	A	VII
9	2-32	Internal ward	06-07-94	A	A	E	H	G	F	A	VIII
10	5-1	Environment	12-07-94	D	B	A	E	H	G	A	IX
11[c]	2-4	BAL hematologic ptt.	30-06-94	C	C	E	E	I	H	A	X
12	2-3	Pancreatitis	10-06-94	C	B	B	I	J	I	A	XI
13[c]	5-26	BAL hematologic ptt.	10-07-94	E	D	F	J	K	J	A	XII
14[c]	6-71	Eye hematologic ptt.	27-05-94	A	C	nd[d]	nd	L	nd	A	XIII
15	2-6	Abdominal drain	30-05-94	A	A	B	B	B	B	A	II
no.of types				5	4	6	10	12	10	1	13

[a]The overall PCR type was based on the number of differing banding patterns when all assays were combined.

[b]CF = cystic fibrosis, BAL = broncho alveolar lavage fluid sample.

[c]Clinical isolates of patients listed in table 2.

[d]nd = not done.

patient were identical, but they differed from a strain isolated from BAL fluid taken before death.

PCR ribotyping identified two types among the *A. flavus* strains. Most of the isolates generated two amplimers (750 and 650 basepairs in length), whereas a subset of strains gave rise to the 750 basepair fragment only. This limited degree of polymorphism did not add to that of the RAPD assays. One of the three American *A. flavus* strains (U3) was the single exception in the sense that only a 450 basepair fragment was synthesized. We assume that this particular strain does not represent *A. flavus.*

Comparing isolates of A. fumigatus. Results of RAPD analysis for the fifteen strains studied are shown in Figure 1; the interpretation is summarized in Table 5. Nearly all isolates of *A. fumigatus* could be differentiated by RAPD analysis using different primers. One RAPD type (overall RAPD type no. II) was found three times, all other combinations were unique. No RAPD identity between environmental and clinical isolates was found. The PCR ribotyping assay resulted in the amplification of a 1200 basepairs long DNA fragment for all the strains, thereby unambiguously identifying the species *A. fumigatus.* *General remarks.* Figure 2 highlights that in the RAPD patterns, species specific DNA fragments (present in all lanes) could be observed. In this way isolates of *A. flavus* and *A. fumigatus* could be discriminated quite easily. This was further confirmed by additional experiments using various other primers (figures not shown). From the overall number of types detectable per primer species it can be concluded that for both *A. flavus* and *A. fumigatus* combined application of primers 5 and 6 results in adequate demonstration of genetic polymorphisms, even though the use of additional primers resulted in further differentiation.

DISCUSSION

Nosocomial aspergillosis still poses a significant clinical problem.[2,4,16] Although PCR tests for detection of fungi have been described recently, this does not yet alleviate the clinical burden.[17,18]

At present PCR techniques appaer to be more useful for the typing of fungal isolates.[19,20] Conclusions concerning relatedness of clinical and environmental isolates have often been based on biochemical and species similarities only. Using these parameters one would have concluded that the apparent outbreak of invasive aspergillosis encountered in the summer of 1994 in the University

Hospital Rotterdam, was due to at least two different species, and that clinical and environmental strains of each species might be identical. More information and stronger evidence for the relatedness of clinical and environmental strains can be derived from analyzing their genomes. For this purpose specialized PCR techniques are currently being introduced in the clinical mycology laboratory.[11,12,19-23] In the present study RAPD assays were applied for the analysis of an outbreak of invasive aspergillosis due to both *A. flavus* and *A. fumigatus*.

Before using RAPD analysis in epidemiologic surveys one has to evaluate the extent of the differences in DNA banding patterns that can be generated. The present report showed that RAPD analysis could discriminate between strains of *A. flavus* and *A. fumigatus*. Strains of *A. flavus* could also be discriminated in this way, but additional discrimination was needed. In preliminary assays with 24 primers and various combinations thereof, five additional primers, which increased the degree of genetic heterogeneity, were selected. Differences between PCR fingerprints obtained for different strains of *A. flavus* appeared smaller than those obtained for strains of *A. fumigatus*. This may indicate more limited chromosomal variation in the former species or a different genome composition requiring other primers. This phenomenon was previously described for *A. nidulans*, for which species the spread of a single clone in extensive areas was reported in a surveillance study.[24]

Combination of different RAPD assays discriminated between nearly all strains of *Aspergillus* spp. In contrast, PCR ribotyping presented *A. fumigatus* as a homogeneous group: only a single amplimer was generated. In case of *A. flavus* two ribotypes were detected. It has recently been proposed that PCR ribotyping may present a broad spectrum strategy suited for typing of all sorts of bacterial species.[25] However, the resolution of this procedure is unacceptably low for *Aspergillus* spp. typing studies.

Using RAPD analysis, all patients' strains of *A. flavus* appeared to be genetically different. RAPD analysis of the whole collection of environmental and nosocomial strains of *A. flavus*, identified one specific genotype repeatedly among the nosocomial strains. These identical patterns were not caused by a lack of discriminative power of the RAPD technique, for several strains of *A. flavus* collected in other laboratories could be discriminated with relative ease.
Thus, among the *A. flavus* strains encountered in the University Hospital Rotterdam, a single clone appeared to predominate on the Hematology ward involved in the outbreak. We consider two options as a possible epidemiological explanati-

on. First, it is possible that this particular strain was brought to the hospital by the index patient, from whom it subsequently spread into the hospital environment: the highest concentrations of spores of the common *A. flavus* type were found during the days that the infection was diagnosed in this patient. This possible kind of spread of conidia from a patient, or from patient' materials has not been described previously. Alternatively, it is possible that this patient may have been infected by conidia deriving from an environmental source within or in the immediate vicinity of the hospital. However, no such source could be detected upon detailed screening of the wards involved; no fungi were detected in the air-conditioning systems or on the HEPA-filters. No rotting wood or aspergilli growing on isolation materials were detected. No fungi were grown from the samples taken from above the ceiling plates. This strain may have been present prior to the infection period, but no strains isolated during this period are available. In addition, no construction activities took place within the hospital at the time of this apparent outbreak. The heavy growth of aspergilli from the air samples taken at a nearby demolition site may have had marginal influence. However, at a distance of 75 meters from this site no increase in the numbers of *Aspergillus* spp. spores was found compared to control samples (the hospital is situated at 150 meters from this site).

As we did not find genetic relatedness among the clinical isolates it is unlikely that the outbreak was a result of conidia spreading from one single source within the hospital. This outbreak may have been the result of generally elevated numbers of conidia from several *Aspergillus* spp. clones present in the outside air. Seasonal variation in *Aspergillus* spp. spore density has been reported.[26] Why large numbers of conidia entered the hospital remains unanswered. With renewed instructions for handling the air-conditioning systems of HEPA filtered rooms, a policy to keep windows closed at all times and continued air sampling, it is hoped that subsequent outbreaks can be prevented.

If knowledge concerning the genetic relatedness of clinical and environmental isolates is available early in the course of an apparent outbreak of invasive aspergillosis, it is possible to start searching for a specific cause. Unrelatedness of isolates should lead to investigations on the functioning of the barrier systems, while relatedness of isolates should induce a search for a common source.

Therefore we recommend to genotype and compare nosocomial and environmental isolates of *Aspergillus* spp. obtained during outbreaks of invasive aspergillosis as soon as possible. RAPD analysis then is a truly rapid and reliable tool.

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Chapter 3

Density and molecular epidemiology of *Aspergillus* in air and it's relation to outbreaks of *Aspergillus* infection.

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ABSTRACT

After an outbreak of invasive aspergillosis, a surveillance program for fungal conidia in the air within and outside the hospital was started. *A. fumigatus* isolates were studied for genetic relatedness as were isolates obtained during an episode of fungal contamination of culture media in the microbiology laboratory.

The density of conidia of non-pathogenic fungi in the outside air showed a seasonal variation: higher densities during the summer, lower densities during fall and winter. There was no seasonal variation in the density of Aspergillus conidia. A decreasing gradient existed in densities of conidia outside the hospital, to inside the hospital, to the department of hematology. The outbreak was not caused by an increase in the number of *Aspergillus* conidia in the outside air. The unique presence of *A. flavus* at that time, indicated the presence of a point source, possibly even a patient or patient materials.

Genotyping of *A. fumigatus* isolates, showed that at the department of hematology, clonally related isolates were present during more than one year. Clinical isolates of *A. fumigatus* obtained during the outbreak, could be discriminated from these persistent clones. *A. fumigatus* isolated during the laboratory contamination were all identical.

We conclude that knowledge of the epidemiology of *Aspergillus* species is important for the development of strategies to prevent invasive aspergillosis. Both the natural density of *Aspergillus* spores and construction activities in a hospital contribute to increased aerial spore concentrations and are causally related to an increased risk of infection for susceptible patients. RAPD-fingerprinting helps to elucidate the complex epidemiology.

INTRODUCTION

Aspergillus species are widely distributed fungi which' conidia are present in the outside air year round, without seasonal influences in conidial density.[1] After inhalation of their conidia, *Aspergillus* species can cause various forms of disease of which invasive infections in immunocompromised patients are the most serious.[2] Despite early treatment with high dosages of amphotericin B, these infections remain associated with high morbidity and mortality.[3] Small outbreaks of aspergillosis have been reported, as have been pseudo-outbreaks due to contamination of culture media.[4-8] Elucidation of the complex epidemiology in such cases requires detailed molecular typing studies. Randomly amplified polymorphic DNA (RAPD) analysis has proved to be a PCR-fingerprinting procedure that can be applied to the typing of fungal isolates.[9-12]

After an outbreak of aspergillosis (caused by *A. fumigatus* and *A. flavus*) in our hospital, we started a surveillance program for fungal conidia in the air within and in the direct surroundings of the hospital.[4] Isolates of *A. fumigatus* obtained from air-samples from the department of hematology were studied for their genetic relatedness. Furthermore, after the surveillance was completed, we experienced an episode of fungal contamination of culture media in the microbiology laboratory which was also studied by molecular typing, using RAPD analysis.

This study was performed to address three questions. First, we wanted to improve our knowledge on the aerobiology of fungi in the environmental air inside and outside of our hospital. Secondly, we wanted to investigate whether the density of conidia at the time of an outbreak of invasive aspergillosis was abnormally high. Finally, we investigated the role of genotyping fungal isolates in elucidating the epidemiology of *Aspergillus* species.

METHODS

Air sampling. During the surveillance period (july 1994 to september 1995) serial air-samples of 1 cubic meter each, were taken with a Surface Air System (SAS)-sampler (PBI international, Milan, Italy) containing Sabouraud agar plates. On each occasion, air samples were taken from the four High-Efficiency Particulate Air (HEPA) filtered rooms and eight other sites within the hematology ward, which department is physically closed by doors. In addition, samples were taken from four generally accessible sites elsewhere within the hospital (general medicine wards and corridors between wards) and four fixed places outside the hospital.

Air-samples were taken to detect *Aspergillus* species as well as non-pathogenic fungi at the same time and further processed. Initially, air-samples were taken twice a week. After two months, further samples were taken once a week.

To determine the density of conidia in the air of the microbiology laboratory, air-samples were taken at several places during and one month after an episode of increased incidence of fungal contamination of culture media.

Identification of fungal isolates. Sabouraud agar plates from the SAS-samplers were incubated at 22°C and 37°C to examine growth of non-pathogenic and pathogenic fungi, respectively. After 48 h incubation at 37°c and after 120 h incubation at 22°C, respectively, Sabouraud agar plates were studied for fungal growth. Species growing at 22°C were counted and not identified further on a regular basis. Species growing at 37°C were identified on the basis of culture characteristics and morphology of conidiophores and conidia. The Sabouraud plates of samples from the microbiology laboratory were only incubated at 37°C to examine growth of *Aspergillus* species.

Collection of fungal isolates. Isolates of *Aspergillus* species obtained from the department of hematology were propagated on fresh Sabouraud media and subsequently stored at -70°C in Brain Heart Infusion (BHI) broth containing 10% glycerol. Isolates of *Aspergillus* species from other departments and from outside the hospital were counted and identified but not stored.

From the contaminated agar plates in the microbiology laboratory, isolates macroscopically suspected to be *A. fumigatus* were collected. Further isolates were obtained from the air-samples taken in the laboratory during the contamination period and one month later. Species were identified as described above.
Isolates of *A. fumigatus* of patients with invasive aspergillosis nursed in the hematology and surgical wards of the Erasmus University Medical Center Rotterdam were used to monitor the discriminative power of the RAPD-assay as published previously.[4]

Fungal DNA isolation: Strains were inoculated in 25 ml Sabouraud maltose medium, containing 4 mg/kg gentamicin, and incubated at 37°C for 72 h until abundant mycelial growth was observed. While working in a safety cabinet, the entire thallus was collected in a porcelain bowl, frozen under liquid nitrogen and grounded with a pestle. Between 10-25 ml lysis buffer (0.1 M Tris-HCl pH 6.4, 40 mM EDTA pH 8.0, 1% Triton X-100, 4 M guanidium isothiocyanate) was added and the suspension was put on ice. One ml of the suspension was centrifuged for 5 minutes (15,000 rpm). To the supernatant 100 µl of a Celite suspension (200 mg/ml) (Aoroa organics, Grel, Belgium) was added and this suspension was

shaken vigorously by hand. The pellet was washed in a second lysis buffer (0.1 M Tris-HCl pH 6.4, 4M guanidium isothiocyanate), ethanol 70% and acetone, respectively.[13] After drying, the pellet was resuspended in 150 µl of 10 mM Tris-HCl pH 8.0 and incubated at 56°C for 10 minutes. Approximately 125 µl of the supernatant was collected. The DNA concentration was estimated by electrophoresis of DNA-containing aliquots through 1% agarose-gels, run in 0.5 x TBE in the presence of ethidium bromide, and comparison with the staining intensities of known amounts of bacteriophage lambda DNA.

PCR-mediated DNA fingerprinting: DNA typing by RAPD was performed exactly as described previously.[14,15] The enterobacterial repetitive intergenic consensus primers ERIC-1 and ERIC-2 were employed for they had shown to discriminate well between epidemiologically non-related isolates in earlier assays.[4] The resulting banding patterns were indexed by capital lettering and even a single band difference led to a different letter code. Differences found in ethidium bromide staining intensities were ignored. Banding patterns were interpreted visually by two independent persons. Reproducibility and reliability were assessed as suggested previously.[12]

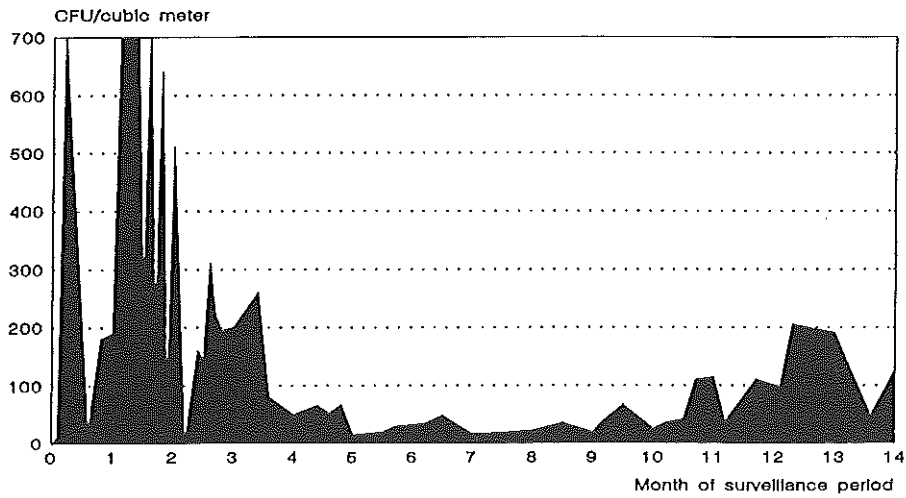
RESULTS

The results section is divided in two parts. The first part describes the results of the year-long surveillance of fungi in air and the results of the molecular analysis of the isolated *Aspergillus* strains during this period. The second part describes the isolation and molecular analysis of the *Aspergillus* strains during a period of laboratory contamination.

Year-long surveillance of fungi in air.

The density of fungal spores in air was monitored on a weekly basis from july 1994 to september 1995. The results of the Colony Forming Unit (CFU) counts of non-pathogenic fungi are shown in Fig. 1. During the first two months of the surveillance period, a median of more than 400 Colony Forming Units (CFU) per cubic meter was counted in the air outside the hospital. In air samples from within the hospital, but outside the hematology ward, this number was significantly lower (32 CFU/m^3), while samples from the hematology ward itself showed a median of 7 CFU/m^3. Air samples from HEPA filtered rooms showed very low fungal densities (<2 CFU/m^3). During the next two months (fall/beginning of winter), the density of non-pathogenic fungal CFU declined outside as well as

1a


CFU/cubic meter
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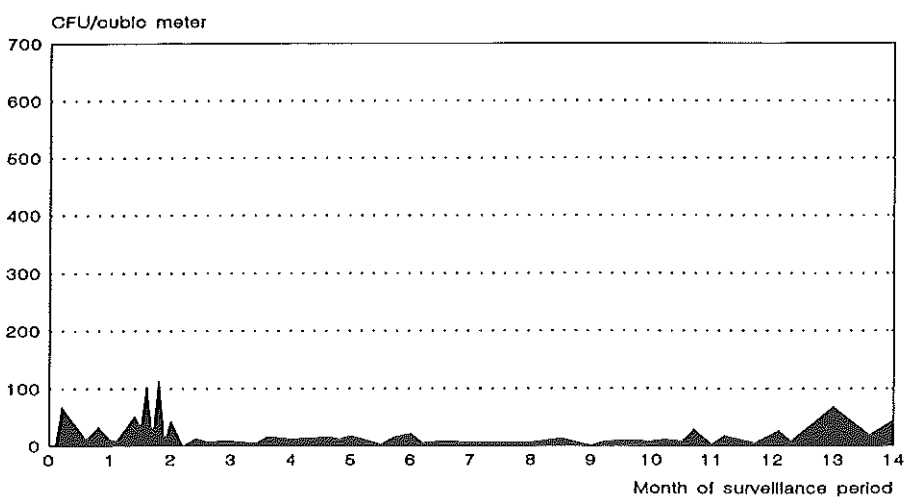
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Month of surveillance period

1b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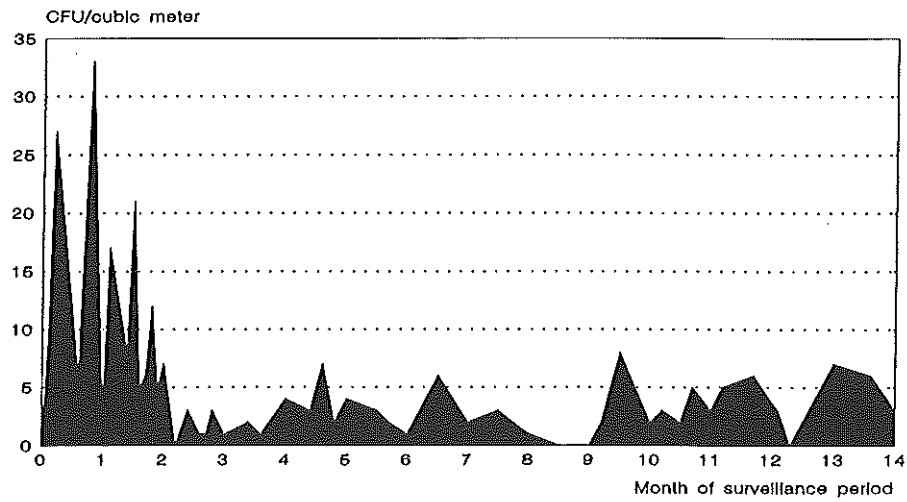
Month of surveillance period

1c



1d

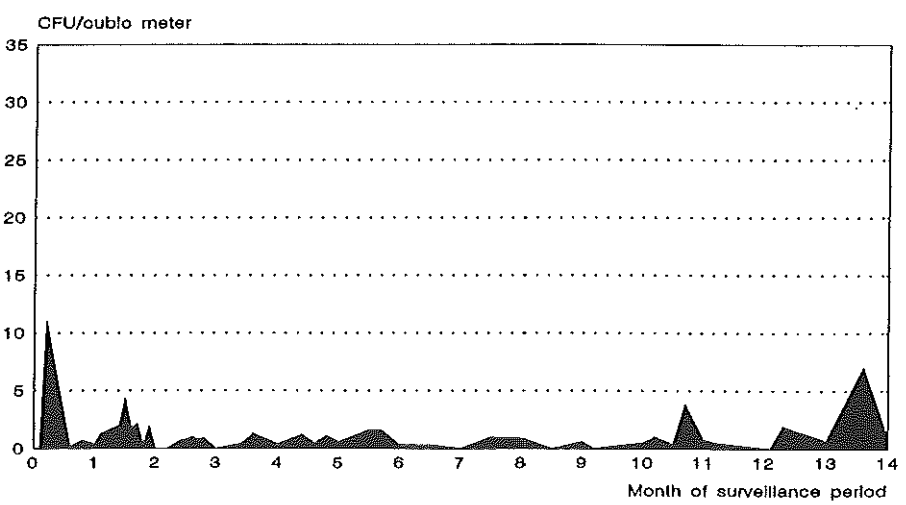
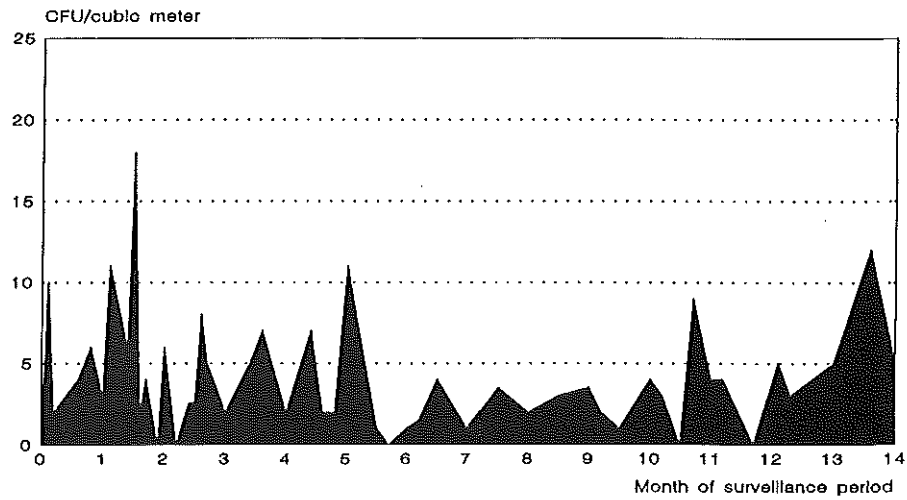


Figure 1. *Results of a surveillance of conidia of non-pathogenic fungi in the outside air (1a), air within the hospital outside (1b) and within the hematology ward (1c) and air in HEPA-filtered rooms (1d). Surveillance started in july 1994 and ended in september 1995.*

2a


CFU/cubic meter
25
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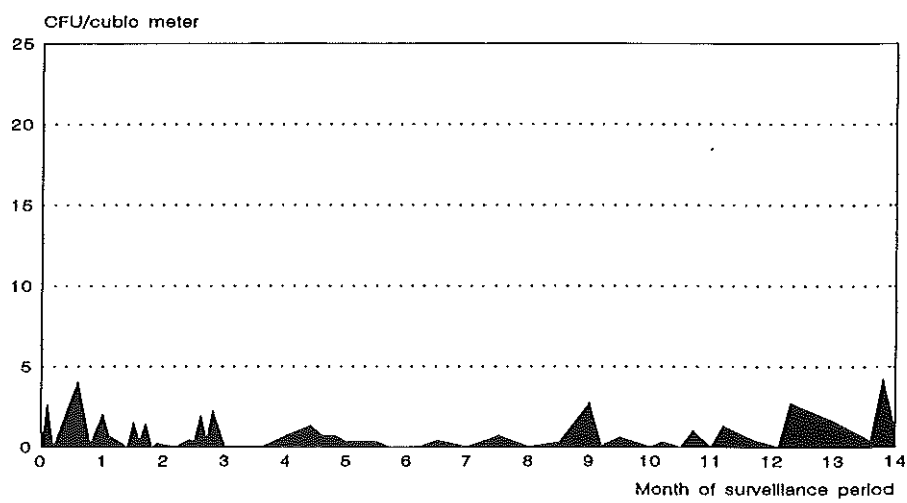
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Month of surveillance period

2b


CFU/cubic meter
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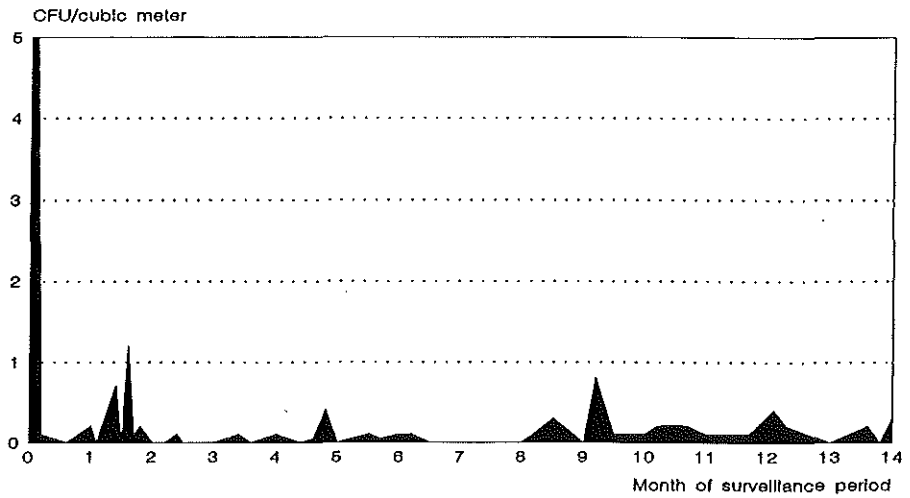
Month of surveillance period

2c



2d

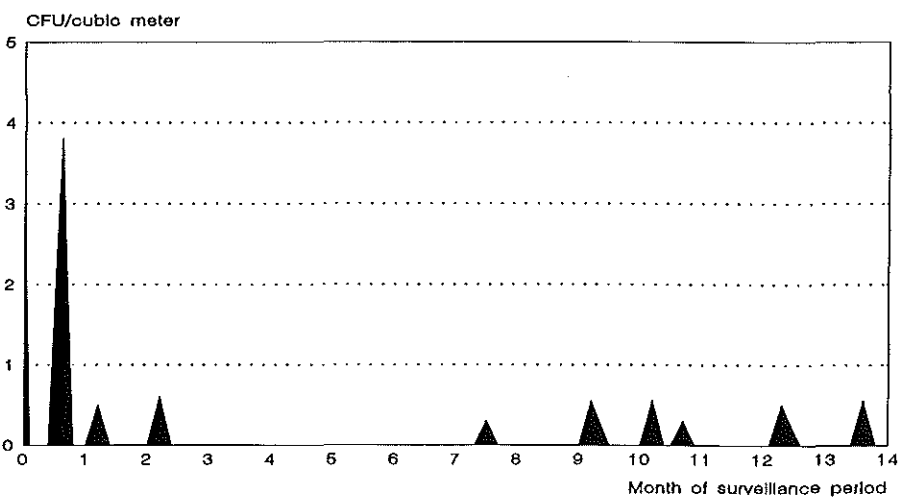


Figure 2. *Results of a surveillance of conidia of Aspergillus spp. in the outside air (1a), air within the hospital outside (1b) and within the hematology ward (1c) and air in HEPA-filtered rooms (1d). Surveillance started in july 1994 and ended in september 1995 (0 to 14 on the x-axis).*

inside the hospital, but the same decreasing gradient between the locations outside versus inside the hospital remained present. The numbers of CFU during winter and spring were low. In the summer months at the end of the surveillance period, the numbers of CFU increased again, but not to the levels observed during the preceding summer (Fig. 1). During the first weeks of sampling, fungi were identified, which showed that the majority (>80%) of the isolates belonged to *Cladosporium* species.

The results of the surveillance of *Aspergillus* species are given in Fig. 2. During the surveillance period the density of aspergilli in the outside air was relatively constant; only from january to april, the number was somewhat lower. The same was true for the number of CFU within the hospital outside the department of hematology. Within the confinement of the department of hematology and in their HEPA-filtered rooms, very few conidia of *Aspergillus* (<1 CFU/m^3) were found, except at the very beginning of the surveillance period.

Most of the isolates from samples of the outside air were identified as *A. fumigatus* (>90%) and *A. niger* (5%). The same species were also found within the hospital, although in the first two months *A. flavus* was isolated more often, especially from the department of hematology. Because of the high numbers of *A. flavus* found in the HEPA-filtered rooms in this period and the fact that aspergilli were only rarely isolated afterwards, *A. flavus* was overall the most frequently isolated species at this particular site in the hospital.

Table 1. *A. fumigatus clones, isolated at the hematological department over a 14-month period.*

Date of isolation		Source	Genotype using PCR primer		Overall genotype
			ERIC-1	ERIC-2	
1994					
june	4	hepa 87	A	A	I
	8	room 92	B	B	II
	19	room 96	B	B	II
	24	room 75	C	A	III
august	3	room 72	A	A	I
	8	room 73	A	A	I
	15	room 96	B	B	II
	22	corridor	A	A	I
	29	room 96	A	A	I
august	31	room 96	A	A	I
september	9	room 88	C	A	III
	12	hepa 91	B	B	II

Table 1. *(continued)*

Date of isolation		Source	Genotype using PCR primer		Overall genotype
			ERIC-1	ERIC-2	
	12	hepa 89	A	A	I
	22	hepa 93	C	A	III
october	26	corridor	B	A	IV
	31	room 88	A	A	I
	31	room 74	C	A	III
november	9	examination room	B	A	IV
	16	room 73	B	A	IV
	23	room 96	C	A	III
1995					
january	4	room 73	A	A	I
	20	room 88	C	A	III
	20	room 93	B	B	II
march	10	room 88	A	A	I
	10	hepa 87	B	A	IV
april	20	room 96	A	A	I
	25	hepa 89	C	A	III
may	3	room 96	B	B	II
	10	room 74	A	A	I
	10	room 84	B	B	II
	16	room 84	B	A	IV
	16	room 82	B	B	II
	30	room 88	A	A	I
	30	room 96	C	A	III
	30	hepa 89	A	A	I
june	7	room 84	D	C	V
	14	room 75	A	A	I
	21	room 75	A	D	VI
	27	room 95	A	A	I
july	26	hepa 93	B	A	IV
august	9	room 75	A	A	I
	23	room 84	B	B	II
september	8	hepa 91	E	E	VII
	20	room 95	B	A	IV
1994		clinical patient	F	F	VIII
1994		clinical patient	G	G	IX
1994		clinical patient	F	H	X
1994		clinical patient	I	I	XI
1994		clinical patient	I	I	XI
1994		clinical patient	J	G	XII

Note: The overall PCR type is based on the banding patterns when both assays were combined.

A. fumigatus isolates from the department of hematology (including HEPA-filtered rooms) were further characterized by means of RAPD (Table 1). The number of different genotypes in air-samples was limited to 7 during the entire

surveillance period. Only three genotypes were found during the first four months; a fourth genotype was introduced in the fourth month and remained present during the remainder of the surveillance period. In the last three months, the number of *A.fumigatus* clones increased, with three new genotypes being introduced. No correlation between rooms and genotypes was found. All clinical isolates of *A. fumigatus* could be differentiated from the environmental isolates. Also, isolates from different patients could be discriminated from each other.

Table 2. *Clones of A. fumigatus clinical isolates and isolates contaminating culture media and air in the medical microbiology laboratory.*

Date of isolation	Source	Genotype using PCR primer		Overall genotype
		ERIC-1	ERIC-2	
may 1994	Clinical patient	-	K	XIII
june 1994	Clinical patient	K	L	XIV
june 1994	Clinical patient	L	M	XV
june 1994	Clinical patient	M	N	XVI
291095	agar plate MML	N	O	XVII
291095	agar plate MML	N	-	XVII
301095	agar plate MML	N	O	XVII
301095	agar plate MML	N	O	XVII
311095	agar plate MML	N	O	XVII
011195	agar plate MML	N	O	XVII
011195	air sample MML	N	O	XVII
011195	air sample MML	N	O	XVII
011195	air sample MML	O	P	XVIII
011195	air sample MML	N	O	XVII
011195	air sample MML	N	O	XVII
011195	air sample MML	N	O	XVII
011195	air sample MML	N	O	XVII
061295	air sample MML	N	P	XIX
061295	air sample MML	N	P	XIX
061295	air sample MML	N	O	XVII
061295	air sample MML	N	O	XVII
061295	air sample MML	N	O	XVII

Note: The overall PCR type was based on the number of differing banding patterns when both assays were combined. MML = Medical Microbiology Laboratory.

Laboratory contamination.

During renovation of the air-conditioning system in the corridor and rooms directly opposite to the microbiology laboratory many cultures became contaminated with

A. fumigatus, despite several precautions (closed doors, air-corridors, intensified cleaning). Contamination was detected on all types of agar plates and in all sorts of materials (even in blood culture bottles). Within a period of 4 days, more than 200 agar plates became contaminated. From these plates, 27 isolates of *A. fumigatus* were collected and further analyzed by RAPD.

Four days after the contamination of the media had become apparent, air-samples were taken. All samples (n=11) showed more than 10 CFU *A. fumigatus* per cubic meter of air. From each of these samples one colony of *A. fumigatus* was likewise genotyped by PCR. One month after the initial contamination period, new air-samples still showed *A. fumigatus* to be present in the air, although in lesser quantities (0-5 CFU per cubic meter). From these samples, five isolates were further analyzed.

All isolates obtained from contaminated cultures displayed the same genotype. Six of these isolates were randomly chosen to be compared with isolates obtained by air sampling, and the clinical isolates used before. The results of this analysis is shown in Table 2. The genotypes of all isolates obtained from the culture media and all except one isolate from air-samples taken during the contamination period, were identical. Three of the five isolates obtained one month later, still had the same genotype. All isolates from the laboratory could be discriminated from the clinical isolates.

DISCUSSION

The numbers of conidia of non-pathogenic and pathogenic fungi detected in this study outside showed a variation that is compatible with surveillances described before: higher numbers during summer, lower numbers during fall and winter.[1,16] There was a clear difference between the peak numbers of conidia during the two summers which were included in the surveillance period. This difference might be due to differences in weather conditions. A very wet period followed by hot dry weather preceded the period of high conidial numbers in 1994. There was almost no seasonal influence on the number of aspergillus conidia.[17] We found a decreasing gradient in the density of conidia from outside the hospital, to inside the hospital, and from open areas within the hospital to the closed department of hematology. The lower density of fungal conidia on the department of hematology may be the result of the increased level of environmental isolation employed at this department (closed entrance doors, windows that can not be opened, no plants or flowers are allowed in, etc). An even higher level of isolation exists

within the HEPA-filtered rooms which resulted in very low numbers of conidia, usually below the advised threshold of air-contamination for such rooms.[18] Only during summer months air-samples from HEPA-filtered rooms showed more conidia of non-pathogenic fungi, probably as a result of a higher density present in the outside air. Because conidia of non-pathogenic fungi are present in much higher densities compared to conidia of pathogenic fungi (10 to 20 times higher densities), enumeration of the former conidia can be conveniently used to control the effectiveness of barrier and filtration systems. During the surveillance period the number of conidia of non-pathogenic fungi indicated that the barrier and filtration systems provided in the HEPA-filtered rooms worked properly.

The gradient found for conidia of non-pathogenic fungi, was also present when only conidia of *Aspergillus* species were counted. Within the hematology ward and the HEPA-filtered rooms, however, the numbers of these conidia were, on two occasions, rather high, during and just after the outbreak of invasive aspergillosis had occurred.[4] Through the subsequent year-long survey, it became clear that during these occasions the density of non-pathogenic fungal conidia in the outside air had also been higher than at any other time during that year. However, the density of *Aspergillus* conidia did not show such a pattern, which made it unlikely that the outbreak of invasive aspergillosis had been due to outside air carrying higher numbers of *Aspergillus* conidia from the outside into the department of hematology. Furthermore, on both occasions, the majority of these conidia were identified as conidia of *A. flavus*, a species which was never found outside the hematology ward. This would indicate the presence of a point source within the department. Because *A. flavus* was also never found after the outbreak, we speculate that the conidia may have been introduced by patients or patient materials. However, during the outbreak, also the density of *A. fumigatus* conidia were higher at the department of hematology and in the HEPA-filtered rooms than any time later. An extensive search for a common source did not reveal such a source. The higher numbers of *A. fumigatus* conidia may have resulted from more frequently opening of windows at other departments at the beginning of the summer. After the beginning of the outbreak, all departments were ordered to keep the windows closed, which may explain for the sudden decrease in the numbers of conidia.

When *A. fumigatus* isolates were genotyped, it was shown that at the department of hematology, clonally related isolates were present. This does not concur with the hypothesis that conidia were being introduced directly from outside into the department. Furthermore, we showed that these clonally related

isolates were present during a period of more than one year and that a newly introduced clone was somehow able to establish itself for almost a year. This suggests that despite our intensive search, one or more common sources of *A. fumigatus* may have been present. Another explanation is that personnel of the hematology department serves as a carrier for conidia from a common source outside the hospital. Unfortunately, personnel was not screened. When we investigated if these isolates were still present more than one year later (january to may 1997), air samples taken at the same locations at the department of hematology did only once show growth of *A. fumigatus*. This isolate was genotypically different from the clones present before (results not shown). Persistence of identical genotypes of *A. fumigatus* was described earlier by Girardin *et al*, who took samples over a period of six months.[19] Although the majority of their isolates could be discriminated, they also occasionally found identical genotypes. This suggests that in their situation a constant introduction of conidia from outside was the source of the strains.

Clinical isolates of *A. fumigatus* obtained during the outbreak, could easily be discriminated from the strains present at the department of hematology. The fact that patients become infected with other strains than those present in the hospital air strongly suggests that patients already are carriers of these strains at the time they are admitted to the hospital. If this is true, the outbreak of invasive aspergillosis and the high number of *Aspergillus* conidia at the same time was merely a coincidence, or the result of the exposure of patients to high numbers of *Aspergillus* conidia before they entered the hospital. This underscores the potential value of the use of prophylactic antifungal regimens in addition to environmental isolation. However, although effective against yeasts, prophylactic antifungal regimens with proven efficacy against moulds still need to be determined.[6,7,20,21]

Genotyping of *A. fumigatus* isolates obtained during the laboratory contamination showed that these isolates were all identical. Whether this contamination was caused directly by the renovation activities opposite to the laboratory with subsequent introduction of genotypically identical isolates of *A. fumigatus*, was not proven. Because the contamination was so extensive, the "pseudo" character of the outbreak was immediately clear and no action was taken towards patients. However, it is not unreasonable to assume that during an episode of laboratory contamination, materials of patients prone to develop invasive aspergillosis become contaminated and show growth of *Aspergillus* species. We demonstrated that in such cases, RAPD may be a very useful

method to determine the clinical relevance of the cultured isolate. For bacterial pathogens, genotyping to determine the clinical relevance of an isolate has been described before.[22]

We conclude that knowledge of the epidemiology of *Aspergillus* species is important for the development of strategies to prevent invasive aspergillosis. Natural variation in the density of aerial spores as well as nosocomial construction activities can contribute to increased aerial spore concentrations. As such, both phenomena may be causally related to increased infection risks for susceptible patients or for pseudo-outbreaks. Genotyping by means of RAPD is very helpful in elucidating this complex epidemiology.

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Chapter 4

Molecular typing of *Cryptococcus neoformans*: taxonomic and epidemiological aspects.

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ABSTRACT

Pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) analysis, serotype, and killer toxin sensitivity patterns of a wide range of saprobic, clinical and veterinary isolates of both varieties of *Cryptococcus neoformans* were examined. *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* differed in chromosomal makeup, RAPD patterns, and killer sensitivity patterns. These results suggest that there are two separate species rather than two varieties. No clear genetic or phenotypic differences were observed among the clinical, saprobic, and veterinary isolates within each taxon. The serotypes differed substantially in their RAPD characteristics. Geographical clustering was observed among the isolates of *C. neoformans* var. *gattii*, but not among the isolates of *C. neoformans* var. *neoformans*. The isolates of each taxon that originated from restricted geographical areas often had identical or similar karyotypes and RAPD patterns, suggesting that clonal reproduction had occurred. The combination of PFGE and RAPD analysis allowed us to distinguish almost all isolates. This combination of techniques is recommended for further research on epidemiological, ecological, and population issues.

INTRODUCTION

Cryptococcus neoformans (Sanfelice) Vuillemin is a zoopathogenic basidiomycetous yeast (teleomorph, *Filobasidiella neoformans* Kwon-Chung) which is usually encountered in the imperfect state. This fungus can cause serious infections, especially in immunocompromised patients. Estimates of the incidence rate in AIDS patients range from 5 to 30%, with the highest numbers occurring in sub-Saharan Africa.[1,2] In human immunodeficiency virus-seropositive individuals, an infection with *C. neoformans* indicates progression to AIDS.[3] The problem is aggravated by the emergence of cryptococcal strains that have become resistant to some of the most widely used antifungal agents.[4-7]

According to the current classification, *C. neoformans* consists of the following two varieties: *C. neoformans* var. *neoformans*, with serotypes A, D, and AD (teleomorph, *F. neoformans* var. *neoformans*) and *C. neoformans* var. *gattii* Vanbreuseghem et Takashio, with serotypes B and C (teleomorph, *F. neoformans* var. *bacillispora* Kwon-Chung).[8-12] The occurrence of recombinations between strains of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* and the demonstration of genetic recombination in the F1 generation have suggested that these taxa are varieties, but no genetic analysis of the F2 generation has been performed.[13,14] In contrast, rather low DNA-DNA reassociation values (range 55 to 63%) have been observed between isolates of the two varieties, which may reflect genetic divergence between the two taxa.[15] The two varieties differ in karyotype, in the number of physiological characteristics (e.g., assimilation of D-proline, D-tryptophan, and L-malic acid), in regulation of creatinine deaminase by ammonia production and in sensitivity to killer toxins of *Cryptococcus laurentii* CBS 139.[16-21]

The two varieties also differ in geographic distribution and habitat. *C. neoformans* var. *neoformans* occurs worldwide, whereas *C. neoformans* var. *gattii* is restricted to the tropics and the southern hemisphere. Both varieties originate from human and animal sources. In the environment *C. neoformans* var. *neoformans* is frequently isolated from bird droppings and is occasionally isolated from substrates like fermenting fruit juice, wood, and air.[2,22,23] Saprobic isolates of *C. neoformans* var. *gattii* are usually associated with *Eucalyptus* species, but have been isolated from bat guano as well.[12,24-27] Differentiation between the varieties is usually performed on L-canavanine-glycine-bromthymol blue medium or by testing D-proline assimilation.[18,28,29] Nearly all of the AIDS-related *C. neoformans*

infections are caused by *C. neoformans* var. *neoformans*, but the two varieties can cause similar neurological syndromes. To diagnose infections and monitor dispersion of strains, technical instruments for cryptococcal genome scanning have been developed.[30-39] Molecular typing techniques have revealed that there is considerable genetic heterogeneity within the species.[16,39] PCR targeted at ribosomal operons has been used to identify species accurately, but has provided insufficient resolution for epidemiological research.[40] A major step forwards was the discovery of a plasmid isolated from a URA5 transformant of *C. neoformans* that could be used to detect genetic polymorphisms.[38,39] Additional probes have subsequently been developed, and simultaneous use of these probes gave results consistent with the URA plasmid analysis results, thus illustrating the validity of the two typing procedures.[37] Analysis of randomly amplified polymorphic DNA (RAPD) has provided a genetic substructure for each variety, but until now no comprehensive study has included both varieties.[31,31,36,41]

In the present study the genetic diversity of the two varieties of *C. neoformans*, including a wide range of environmental and medical isolates, was analyzed by karyotyping and by performing RAPD analysis with enterobacterial repetitive intergenic consensus (ERIC) primers.[42,43] In addition, serotypes and killer toxin sensitivity patterns were analyzed, and epidemiological features were discussed.

MATERIALS AND METHODS

Fungal strains. Medical and saprobic isolates of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* were obtained from the collections of the Yeast Division, Centraalbureau voor Schimmelcultures (Delft, The Netherlands), the Laboratory of Mycology, Institut of Tropical Medicine (Antwerp, Belgium), the Department of Bacteriology, University Hospital Rotterdam (Rotterdam, The Netherlands), and D. Ellis (Adelaide, Australia). Additional isolates were obtained from the National Institutes of Health (Bethesda, Md.) and the American Type Culture Collection (Rockville, Md.). The varieties to which the strains belonged were confirmed by performing colour reaction tests on L-canavine-glycine-bromthymol blue medium, D-proline assimilation tests and killer toxin sensitivity tests. A total of 92 *C. neoformans* var. *neoformans* strains and 32 *C. neoformans* var. *gattii* strains were studied (Tables 1a and 1b).

Serotyping. The antigens used for production of antisera were prepared

Table 1a. *Origins of the isolates of C. neoformans var. neoformans studied.*

Strain[a]	Origin	sero type	mating type[b]	killer type[c]	RAPD type
CBS 131	Institut Pasteur, Paris France	AD	?	IV	AA
CBS 132	Fermenting fruit juice	D	alfa	II	AA
CBS 464	Laboratoire de Parasitologie, Paris, France	A	?	IV	AA
CBS 879	Ulcerated cheek	A	alfa	II	BB
CBS 880	Unknown	A	alfa	II	BC
CBS 881	36-year old woman	A	alfa	II	BB
CBS 882	Nasal tumor of horse, type strain *Torula nasalis*	D	alfa	II	CD
CBS 884	Brain, The Netherlands	A	alfa	II	BB
CBS 885	Unknown	A	alfa	II	BC
CBS 886	Unknown	A	alfa	II	BB
CBS 887	Unknown	A	alfa	III	BB
CBS 888	Unknown	D	alfa	II	CD
CBS 916	Unknown	A	alfa	II	BE
CBS 918	Dead white mouse	D	alfa	III	CD
CBS 939	Unknown	AD	?	III	AF
CBS 950	Tumor	A	?	III	AF
CBS 996	Blastomycosis in man, Type strain *C. psicrophylicus*	A	alfa	III	BB
CBS 1009	Man	A	alfa	II	BB
CBS 1143	CSF	A	alfa	II	BF
CBS 1144	CSF	A	alfa	III	BB
CBS 1584	Unknown	A	?	IV	AF
CBS 1931	Soil	A	alfa	II	BB
CBS 1932	Soil	A	?	II	BB
CBS 1933	Mastitic cow, US	A	?	II	BC
CBS 1935	Soil	A	alfa	II	BB
CBS 2771	CSF	A	alfa	II	BB
CBS 4194	Spleen, Germany	D	alfa	II	CB
CBS 4572	CSF	A	alfa	II	BB
CBS 4868	Sputum, The Netherlands	A	?	II	BB
CBS 5467	Milk from mastitic cow, Switzerland	D	alfa	III	CD
CBS 5474	Mastitic cow	D	?	III	CD
CBS 5728	Non-meningitic cellulitis and osteomyelitis, US	D	alfa	II	CD
CBS 6885	Bone lesion in man, type strain *F. neoformans*	D	alfa	II	CD
CBS 6886	Droppings of pigeon	D	a	II	CD
CBS 6900	Genetic offspring of CBS 6885 x CBS 6886	D	alfa	II	CD
CBS 6901	Genetic offspring of CBS 6885 x CBS 6886	D	a	III	CD
CBS 6961	Man, Oklahoma	A	alfa	II	DE
CBS 6995	CCF, Illinois	A	a	IV	CD
CBS 6999	Pigeon droppings, Thailand	A	alfa	II	BB
CBS 7000	Pigeon droppings, Denmark	D	a	III	CD
CBS 7779	AIDS patient, Argentina	A	alfa	II	BB
CBS 7812	CSF	A	?	I	BC
CBS 1814	Air, Belgium	AD	alfa	II	CD
CBS 1815	Pigeon droppings, Czechoslovakia	D	alfa	II	CD
CBS 7816	Cuckoo droppings, Thailand	A	alfa/a	II	CD

Table 1a. (continued)

Strain	Origin	sero type	mating type	killer type	RAPD type
RV 26952	CSF, Zaire	A	?	II	BB
RV 46115	Plants, India	A	?	II	BC
RV 46119	Pigeon droppings, India	A	?	II	BB
RV 46129	Pigeon droppings, India	A	?	II	BB
RV 52733	Pigeon droppings, Belgium	D	?	II	AC
RV 52755	CSF, Belgium	AD	?	II	AC
RV 53794	Canary bird droppings, Belgium	D	?	II	AC
RV 55446	House dust, Zaire	A	?	II	BA
RV 55447	Air inside house, Zaire	A	?	II	BC
RV 55451	Cockroach, Zaire	A	?	III	BC
RV 55980	Canary bird droppings, Belgium	A	?	II	BB
RV 56126	CSF AIDS patient, Belgium (visited Haiti)	A	?	II	GF
RV 56883	Canary bird droppings, Belgium	A	?	II	BB
RV 56894	Canary bird droppings, Belgium	A	?	II	BB
RV 58145	Wood, Zaire	A	?	II	BC
RV 58146	Wood, Zaire	A	?	II	EG
RV 59351	Parrot droppings, Belgium	A	?	II	BA
RV 59379	Air in zoo, Belgium	A	?	II	BB
RV 60047	Skin cryptococcosis, Belgium	D	?	II	BC
RV 61756	Man, Belgium	AD	?	II	AA
RV 61790	Man, Belgium (visited Zaire)	A	?	II	BA
RV 62210	CSF AIDS patient, Belgium	A	?	II	BA
RV 62692	Skin cryptococcosis, Belgium	D	?	II	CD
RV 63214	CSF AIDS patient, Zaire	A	?	II	BB
RV 63642	CSF AIDS patient, Brazil	A	?	II	BA
RV 64610	AIDS patient, Rwanda	A	?	II	CE
RV 64612	AIDS patient, Rwanda	A	?	II	BA
RV 65631	CSF, Zaire	A	?	II	BB
RV 65662	Man Portugal, visited Venezuela	A	?	II	FC
RV 66025	Cryptococcoma, Belgium	A	?	II	BC
RV 66055	AIDS patient, Rwanda	A	?	II	BA
RDA 1335	AIDS patient no 1, Rotterdam, The Netherlands	A	?	II	BA
RDA 1340	AIDS patient no 1, Rotterdam, The Netherlands	A	?	II	BA
RDA 1371	AIDS patient no 2, Rotterdam, The Netherlands	A	?	II	BA
RDA 1369	AIDS patient no 3, Rotterdam, The Netherlands	A	?	II	BA
RDA 1373	AIDS patient no 3, Rotterdam, The Netherlands	A	?	II	BA
RDA 1445	AIDS patient no 4, Rotterdam, The Netherlands	A	?	II	BA
RDA 1419	AIDS patient no 5, Rotterdam, The Netherlands	D	?	I	FF
RDA 1549	AIDS patient no 6, Rotterdam, The Netherlands	A	?	II	BA
RDA 1589	AIDS patient no 7, Rotterdam, The Netherlands	A	?	II	B-
RDA 1006	AIDS patient no 8, Rotterdam, The Netherlands	A	?	II	BA
RDA 4092	AIDS patient no 9, Rotterdam, The Netherlands	A	?	II	BA
RDA 4094	AIDS patient no 10, Rotterdam, The Netherlands	A	?	II	BA
RDA 4054	AIDS patient no 11, Rotterdam, The Netherlands	A	?	II	BA
RDA 4091	AIDS patient no 12, Rotterdam, The Netherlands	A	?	II	BA

Table 1b. Origins of the isolates of C. neoformans var. gattii studied.

Strain	Origin	sero type	mating type	killer type	RAPD type
CBS 883	Infected skin,Syntype of *C. hondurianus*	B	?	V	GH
CBS 919	Meningoencephalitis, type strain *T. neoformans* var. *sheppei*	B	?	V	GH
CBS 1622	Tumor	B	?	V	GH
CBS 1930	Sick goat, Aruba	B	?	VII	HI
CBS 1934	Mastitic cow, US	B	?	V	GJ
CBS 2502	Suspected case of tubercular meningitis	B	?	V	GJ
CBS 5757	Unknown	B	alfa	V	GH
CBS 5758	Unknown	C	alfa	VI	DK
CBS 6289	Subculture of type strain RV 20186	B	a	VII	IJ
CBS 6290	Man, Republic of Congo	B	?	V	GJ
CBS 6955	CSF, type strain of *F. bacillispora*, California	C	a	VI	DG
CBS 6956	Sputum, Washington	B	alfa	VIII	IN
CBS 6992	Man	B	alfa	V	GH
CBS 6993	Man, California	C	alfa	VI	DK
CBS 6994	CSF, New Jersey	C	alfa	IX	DG
CBS 6996	Man	B	alfa	VII	EL
CBS 6997	CSF, California	B	alfa	VI	DK
CBS 6998	CSF, Thailand	B	a	X	EM
CBS 7229	Meningitis, Type strain of *C. neoformans* var. *shanghaiensis*, China	B	?	V	GH
CBS 7523	*Eucalyptus camaldulensis*, Australia	B	?	V	GH
CBS 7740	CSF, Punjab, India	B	?	VII	GH
CBS 7741	CSF, Punjab, India	B	?	VII	GH
CBS 7742	CSF, Punjab, India	B	?	VI	GH
CBS 7747	Seedling of olive, Australia	B	?	V	GH
CBS 7748	Air in hollow *Eucalyptus camaldulensis*, Australia	B	?	V	GH
CBS 7749	Bark of *Eucalyptus camaldulensis*, Australia	B	?	V	GH
CBS 7750	Bark debris of *Eucalyptus camaldulensis*, California	B	?	VII	HI
RV 5265	CSF, Zaire	B	?	?	GJ
RV 20186	CSF, Zaire	B	a	VII	GJ
RV 54130	Isolate of *C. neoformans* var. *shanghaiensis*	B	?	VI	GJ
RV 66095	CSF, Brazil	B	?	X	GJ
ATCC 32269	Subculture of type strain of *C. gattii*	B	a	?	GJ
NIH-B-3939	Subculture of type strain of *C. gattii*	B	a	?	GJ

[a]CBS = Centraalbureau voor Schimmelcultures Yeast Division; RV = Institut of Tropical Medicine; RDA = Department of Bacteriology, University Hospital Rotterdam; ATCC = American Type Culture Collection; NIH = National Institutes of Health.
[b]? = not known.
[c]Killer sensitivity types as described by Boekhout and Scozetti.[21]

from the following strains: RV56164 (serotype A), isolated from canary bird droppings in Belgium; RV68038 (serotype D), isolated from wood in Burundi;

RV20185 (=ATCC 32267) (serotype B); and RV 45978 (=ATCC 34880) serotype C. Polyclonal antisera were raised by immunizing female New Zealand White rabbits with heat-killed *C. neoformans* cells. The antisera used for final serotyping in a slide agglutination test were first adsorbed with a mixture of cells of the other serotypes by using the method described by Wilson et al.[44]

Killer toxin sensitivity. Killer toxin sensitivity was analyzed by using protocols described elsewhere.[21] The following killer strains were used: *C. laurentii* (Kufferath) Skinner CBS 139, CBS 7235, and CBS 7857; *C. podzolicus* (Bab'eva et Reshetova) Golubev CBS 7717; *C. humicola* (Daszewska) Golubev CBS 4281; and *F. capsuligenum* Rodrigues de Miranda CBS 4736. Ten sensitivity types were discerned.[21]

PFGE analysis. The cells used for the pulsed-field gel electrophoresis (PFGE) were grown on 1% yeast extract-0.5% peptone-4% glucose broth at 25°C with shaking at 180 rpm. Agarose plugs were prepared as described by De Jonge et al. using Novozym 234 (Novo Industri AS, Bagsvaerd, Denmark).[45] Electrophoresis was performed in a contour-clamped homogeneous electric field (CHEF) DR-II apparatus (Bio-Rad, Veenendaal, The Netherlands) by using the following conditions: 30 h with a ramping pulse time from 100 to 300 s, followed by 40 h with a ramping pulse time from 400 to 600 s. Initially 110 V was applied, but during later experiments 100 V was applied. Gels were made with chromosome grade agarose (Bio-Rad) in 0.5xTBE and were electrophoresed at 12°C. Commercially available plugs of *Saccharomyces cerevisiae* and *Hansenula wingei* (=*Pichia canadensis*) (both obtained from Bio-Rad) were used as molecular size standards. After electrophoresis was completed, the gels were stained with 0.5μg of ethidium bromide per ml for 30 min, destained with distilled water for 30 min, and photographed with UV transilluminator at 300 nm. The sizes of chromosomes were calculated by comparing running distances with a plot of running distance versus chromosome size of the standards. Karyotypes were also compared by using the program Gelcompar (Applied Maths, Ghent, Belgium).

RAPD analysis. DNA for the RAPD analysis was isolated from colonies grown for 2 or 3 days on solid Sabouraud medium at 30°C. Cells were harvested by suspending them in 1 ml of 20 mM sodium citrate (pH 5.8)-1 M sorbitol containing 10 mg of Novozym 234 per ml. The suspension was incubated at 37°C for 2 h, and spheroplasts were collected by centrifugation. DNA was isolated by lysing the spheroplasts in guanidium isothiocyanate-containing

buffers, and this was followed by affinity purification with Celite (Acros, Geel, Belgium).[46] DNA was dissolved in 10 mM Tris-HCl (pH 8.0)-1 mM EDTA and stored at -20°C. Concentrations were determined by comparing the ethidium bromide staining intensities of aliquots with the staining intensities of known amounts of bacteriophage lambda DNA. The RAPD analysis was performed by using the following cycling parameters: predenaturation at 94°C for 4 min, followed by 35 cycles consisting of 1 min 94°C, 1 min 25°C, and 2 min 74°C.[42,47] Approximately 50 ng of DNA was amplified by using primers ERIC1 and ERIC2 (50 pmol in 100 μl). The DNA band patterns obtained with these two primers were inspected visually and were designated by using uppercase letters. If patterns obtained with the same primer differed in more than two bands, a novel type was defined.

RESULTS

Serotyping. The serotype data are listed in Tables 1a and 1b. A serological comparison of the clinical, veterinary, and saprobic isolates (Table 2) showed that most of the clinical and saprobic isolates of *C. neoformans* var. *neoformans* were serotype A organisms, but four of the five veterinary isolates were serotype D organisms. All of the saprobic and veterinary isolates and most of the clinical isolates of *C. neoformans* var. *gattii* were serotype B organisms.

Table 2. *Distribution of serological types in clinical, saprobic, and veterinary isolates of C. neoformans var. neoformans and C. neoformans var. gattii*

Type of isolate	*C. neoformans* var. *neoformans*				*C.neoformans* var. *gattii*		
	No.	(% serotypes)			No.	(% serotypes)	
		A	D	AD		B	C
Saprobic	25	72	24	4	5	100	0
Clinical	34	83	13	4	19	85	15
Veterinary	5	20	80	0	2	100	0
Other	1	100	0	0	0	0	0

Killer sensitivity. The killer sensitivity data are shown in Tables 1a and 1b. The two varieties had different killer sensitivity patters. Isolates of *C. neoformans* var. *neoformans* belonged to killer sensitivity types I to IV. About 80% of these organisms were type II organisms, 13% were type III organisms, 4% were type IV organisms, and 2% were not sensitive at all (type I). No clear relationship among serotype, source of isolation, and geography was obeserved. About 43% of the *C. neoformans* var. *gattii* isolates were killer sensitivity type V organisms, 23% were type VII organisms, 20% were type VI organisms, 7% were type X organisms, 3% were type VIII organisms, and 3% were type IX organisms. All six of these killer sensitivity types were represented by the American isolates studied. The African and Asian populations contained fewer killer sensitivity types, and in the Australian population only one type (type V) was identified. The only California isolate studied from *Eucalyptus* sp. was a type VII organism.

Table 3. *Distribution of chromosome numbers among isolates of C. neoformans var. neoformans and C. neoformans var. gattii.*

No. of chromosomal DNA bands	% of *C. neoformans* var. *neoformans* isolates			% of *C. neoformans* var. *gattii* isolates
	clinical (n = 55)	veterinary (n = 5)	saprobic (n = 20)	(n = 25)
9	4			
10	7		10	20
11	22		20	8
12	29	20	35	20
13	15	20	20	40
14	9	40	5	12
15	7	20	5	
16	2		5	
17				
18				
19	2			
20	2			
21	2			

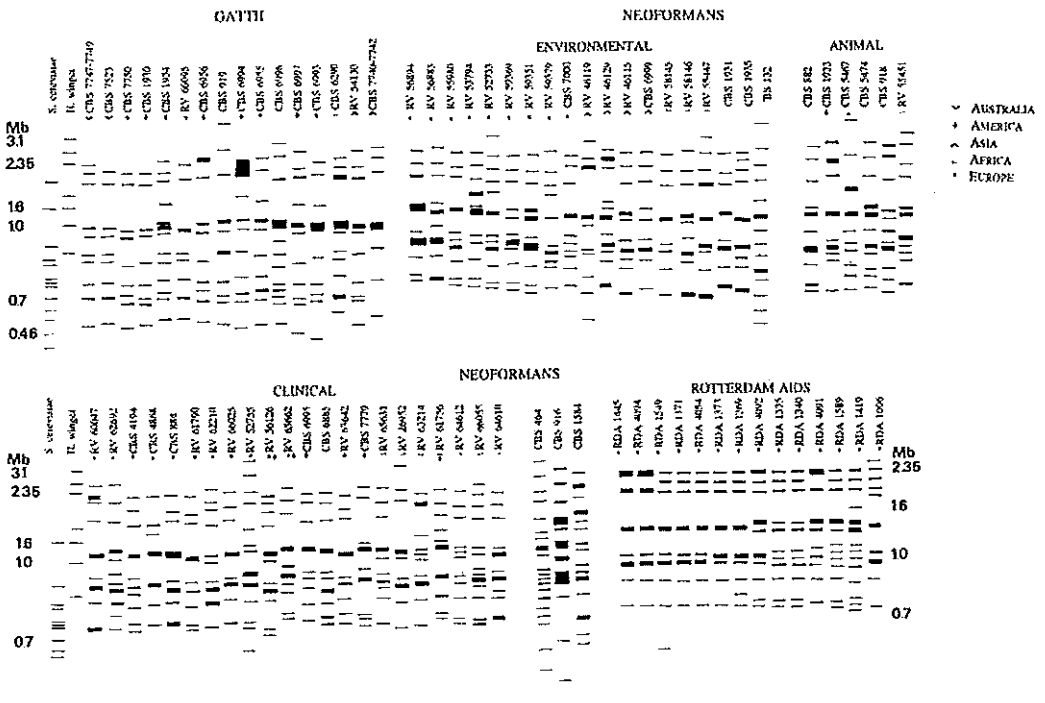

Figure 1. *Schematic representation of karyotypes of C. neoformans. (S. cerevisiae and P. canadensis were the standards used.)*

Karyotyping by PFGE. The karyotype analysis revealed considerable heterogeneity among the isolates (Fig. 1). Most isolates of *C. neoformans* var. *neoformans*, whether they were clinical, veterinary, or saprobic, produced a variety of band patterns; these patterns usually consisted of 11 to 13 bands (range 9 to 21) (Table 3). The size of the individual chromosomes usually ranged between ca. 0.7 and 2.7 Mb, but in some isolates (e.g., CBS 132, CBS 464, CBS 916, CBS 5467, and RV 26952) a larger chromosome (≥3.1 Mb) was present (Fig. 1). In some isolates a smaller chromosome was present; e.g. RV 46119 had a ca. 500-kb chromosome, CBS 950 had a ca. 600-kb chromosome and CBS 916 had a ca. 300-kb chromosome. No consistent differences were apparent among the clinical, veterinary, and saprobic isolates or among serotype A, D, and AD isolates. The overall pattern consisted of four or five to eight to nine bands

between ca. 0.7 and 1.4 Mb, frequently with a doublet or triplet at ca. 1 and 1.4 Mb, and three or four bands between 1.8 and 2.7 Mb. A number of isolates contained a more or less continuous series of chromosomes. This was the case in CBS 132, the type strain of *C. neoformans* var. *neoformans*, and in CBS 464 and RV 52755 (Fig. 1). The genome sizes of selected isolates of *C. neoformans* var. *neoformans* that had no or a few doublet chromosomes varied between 15 and 27 Mb (Table 4).

Table 4. *Estimated genome sizes of selected isolates of C. neoformans.*

Taxon	Strain	Estimated genome size (Mb)
C. neoformans var. *neoformans*	CBS 464	27
	CBS 918	19
	CBS 6885	19
	CBS 7779	15
	RV 52755	22
	RV 59379	18.5
	RV 61756	20
C. neoformans var. *gattii*	CBS 883	17
	CBS 919	16.5
	CBS 1930	15
	CBS 6289	18
	CBS 6955	16
	CBS 6992	16
	CBS 6993	16
	CBS 6996	16
	CBS 6997	18
	CBS 7229	17
	CBS 7748	15
	CBS 7750	14
	RV 54130	17
	RV 66095	12

The lengths of the chromosomes of *C. neoformans* var. *gattii* isolates usually varied between ca. 500 kb and 2.7 Mb (Fig. 1) and the number of

chromosomes ranged from 10 to 14 (Table 3). In some isolates (e.g., CBS 919 and CBS 6289) a larger chromosome (≥3.1 Mb) was present (Fig. 1). The sizes of the smallest chromosomes of most *C. neoformans* var. *gattii* isolates were estimated to vary between ca. 0.5 and 0.6 Mb; the only exception was CBS 6998, whose lowermost band was at ca. 0.75 Mb (data not shown). The overall patterns contained ca. six or seven bands between ca. 0.5 and 1.4 Mb, with a doublet (or triplet) at ca. 1.4 Mb, and two or three bands between ca. 2.0 and 2.4 Mb. All of the serotype C isolates had an additional band at 1.8 Mb, which was present in only one serotype B isolates (CBS 6997). The genome sizes of *C. neoformans* var. *gattii* strains were on the average smaller than the genome sizes of *C. neoformans* var. *neoformans* strains (Table 4). Estimates of sizes based solely on isolates that had no or a few doublet chromosomes varied between ca. 12 and 18 Mb.

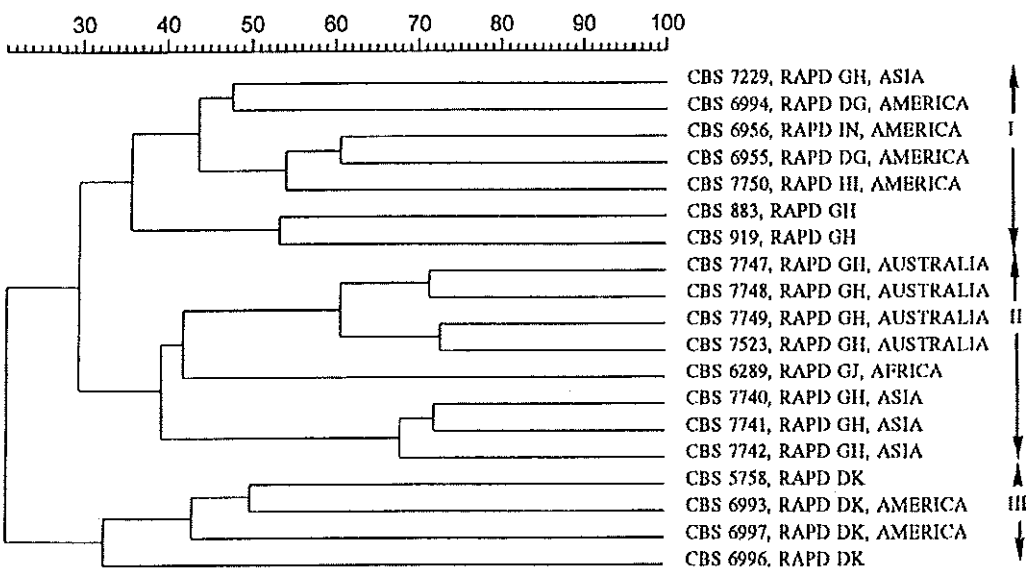

Figure 2. *Unweighted pair group with arithmetic average clustering of C. neoformans var. gattii and correlation with RAPD types. There are three main clusters (Cluster I, II, and III), which correlate well with RAPD types.*

No differentiation was observed between clinical and saprobic isolates.

Three main clusters were identified after a cluster analysis performed with the unweighed pair group method using arithmetic averages (Fig 2.). Cluster 1 contained some of the American isolates, as well as a Chinese isolate; cluster 2 contained Australian, African, and Indian isolates; and cluster 3 contained only American isolates. The saprobic strains from Australia (cluster 2) had similar karyotypes and identical RAPD types. The karyotypes and RAPD patterns of three medical isolates form India (cluster 2) were identical. Also, the karyotypes and RAPD patterns of two meningitis isolates (CBS 7229 and RV 54130) from the Peoples's republic of China and clinical isolate CBS 919 from the United States were similar.

The karyotypes of type strain RV 20186 of *C. neoformans* var. *gattii* and its subcultures stored in different culture collections (CBS 6289 and ATCC 32269) were identical, whereas subculture NIH B-3939 differed in a number of bands. The latter strain lacked the upper and lower bands and had an additional band at ca. 1.8 Mb. The largest chromosome of isolate RV 20186, which was lyophilized shortly after its discovery in 1968, was found to be somewhat smaller than the largest chromosome of a subculture lyophilized in 1986 after multiple rounds of cultivation on agar slants.

RAPD analysis. The complete data set obtained with ERIC1 and ERIC2 is presented and interpreted in Table 1. Sixteen different genotypes occurred in *C. neoformans* var. *neoformans*, resulting in an index of variation (i.v.) of 16/91 = 15.5. However 83% of the *C. neoformans* var. *neoformans* strains belonged to only nine genotypes, which resulted in an i.v. of 11. Eight different genotypes occurred in *C. neoformans* var. *gattii*, resulting in an i.v. of 8/30 = 27. However 77% of the strains belonged to only three genotypes (i.v. of 13).

Comparisons of genotypes and serotypes, origins, and geographical distribution are presented in Tables 5 through 7. No clear differences among the clinical, saprobic, and veterinary strains were apparent (Table 5). The largest number of RAPD types occurred among strains of clinical origin, but differences in the i.v.'s appeared to be insignificant. Four of the five veterinary isolates of *C. neoformans* var. *neoformans* (80%) were RAPD type CD organisms, and 80% of the isolates from AIDS patients were type BA organisms. Most of the serotype B strains differed from the serotype C strains; the only exception was one RAPD type DK strain (Table 6). About 70% of the serotype D strains, but only 3% of the serotype A strains, were genotype CD organisms. Strains of *C. neoformans* var. *neoformans* that originated from different continents did not differ widely in

genetic structure, whereas the strains of *C. neoformans* var. *gattii* exhibited a geographic substructure (Table 7). The single Australian RAPD type, also occurred in Asia, but was absent in Africa and the Americas. The Asian, African, and American populations were linked by RAPD type GJ. About 80% of the American isolates exhibited RAPD types (DG, DK, HI, and IN) that did not occur on the other continents.

Table 5. *Distribution of RAPD types among clinical, saprobic, and veterinary isolates of C. neoformans.*

Type of isolates	*C. neoformans* var. *neoformans*		*C. neoformans* var. *gattii*	
	RAPD type	No. of strains	RAPD type	No. of strains
Clinical	BA	17	GH	7
	BB	13	GJ	6
	CD	4	DG, DK	2
	BC	3	EL, EM, IN	1
	AA, AC, AF, BF, CB, CE, DE, FC, FF, GF	1		
Saprobic	BB	10	GH	4
	CD	5	HI	1
	BC	4		
	BA	3		
	AC	2		
	AA, EG	1		
Veterinary	CD	4	HI, GJ	1
	BC	1		

Local isolates from AIDS patients. The Rotterdam isolates from AIDS patients had rather homogeneous karyotypes and RAPD types (Fig. 1 and Table 1). All of the strains except one were RAPD type BA organisms. Isolate RDA

Table 6. *Distribution of RAPD types among serotypes of C. neoformans.*

Serotype	RAPD type	No. of strains
A	BB	23
	BA	20
	BC	9
	AF, BE, CD	2
	AA, BF, CE, DE, EG, FC, GF	1
D	CD	13
	AC	2
	AA, BC, CB, FF	1
AD	AA	2
	AC, AF, CD	1
B	GH	12
	GJ	7
	HI	2
	DK, EL, EM, IN	1
C	DK, DG	2

Table 7. *Geographic distribution of RAPD types of C. neoformans.*

Location	*C. neoformans* var. *neoformans*		*C. neoformans* var. *gattii*	
	RAPD type	No. of strains	RAPD type	No. of strains
America	CD	3	GJ, DG, DK, HI	2
	BA, BB, BC, DE	1	IN	1
Europe	BA	16		
	BB	6		
	CD	5		
	AC	3		
	BC	2		
	AA, CB, FC, FF, GF	1		
Africa	BA, BB, BC	3	GJ	4
	CE, EG	1		
Asia	BB	3	GH	4
	BC, CD	1	EM, GJ	1
Australia			GH	4

1419 differed in RAPD type, karyotype, serotype, and killer sensitivity type. Two isolates obtained from the same patient had identical karyotypes and RAPD types. However, the two isolates from another patient differed slightly in the penultimate band. Two series of isolates (RDA 1371, RDA 1373, RDA 1549, and RDA 4054 and isolates RDA 1445 and RDA 4094) could not be differentiated by combination of karyotyping and RAPD typing. PFGE revealed more genetic heterogeneity among the Rotterdam isolates than RAPD typing revealed.

DISCUSSION

Recently, several genotyping techniques have been used to detect genetic variation in *C. neoformans*.[4,16,30-33,35,36,39,41,48,49] In these studies usually a limited number of isolates (e.g., isolates originating from a restricted geographic area or isolates belonging to only one of the varieties) have been studied. In the present study, in which a wide variety of isolates of both *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* were studied, the observed differences in genetic makeup, mycotoxin sensitivity patterns, and serology between the two varieties bring into question the conspecific status of these taxa. If the two entities interbreed, we do not understand how the genetic differences are maintained, as homogenization of the population would be expected. However, if reproduction is clonal, as suggested previously, the presence of mutually exclusive genetic patterns suggests that different species are involved.[30] Therefore, because of intermediate DNA-DNA hybridization values, we have concluded that two separate species may be involved.[15] If this turns out to be true, the binomials *Cryptococcus bacillisporus* Kwon-Chung et Bennett (synonym, *C. neoformans* var. *gattii* Vanbreuseghem et Takashio) and *Filobasidiella bacillispora* Kwon-Chung should be used for the second taxon in its anamorphic and teleomorphic stages, respectively.

Karyotype analysis has revealed strain-specific band patterns.[33,35] Groups of strains with similar or identical karyotypes usually have identical RAPD types. The first analysis of the *C. neoformans* genome revealed 12 or 13 chromosomes in the type strain of *C. neoformans* var. *neoformans*, CBS 132.[45] Somewhat later, three different karyotype patterns were observed (one for serotype A, one for serotype D and one for serotypes B or C isolates).[50] Perfect et al., however, found considerable variation in the band patterns within all four serotypes.[51]

Wickes et al. observed differences between the karyotypes of *C. neoformans* var. *gattii* and *C. neoformans* var. *neoformans*, and this was largely confirmed by our results.[16] However, some isolates of *C. neoformans* var. *neoformans* (e.g., CBS 132, CBS 464, CBS 916, and CBS 1584) contained smaller chromosomes (up to ca. 0.3 Mb), which fell into the size range of *C. neoformans* var. *gattii* chromosomes. When used with caution, karyotypes may provide estimates of genome size. A serious problem in estimating genome sizes on the basis of karyotypes is that comigrating bands may occur, and different estimates are used for individual chromosomes. Perfect et al. estimated that the genome size of *C. neoformans* is between 15 and 17 Mb, with the number of bands ranging from 10 to 12.[51] Later Wickes et al. calculated considerably larger genome sizes, ca.21 to 24.5 Mb, with 13 chromosomes on average in *C. neoformans* var. *gattii* and 12 chromosomes in *C. neoformans* var. *neoformans*. Our results revealed considerable variation in genome size in both varieties, suggesting that there may be differences in ploidy and/or aneuploidy within the species. The genome size of *C. neoformans* var. *neoformans* was found to be somewhat larger than the genome size of *C. neoformans* var. *gattii*.

We observed some differences among the karyotypes of the type strains of *C. neoformans* var. *gattii* maintained in different culture collections. These differences were supported by DNA fingerprinting results, but our RAPD patterns were identical.[52] The following two explanations may account for this phenomenon: 1) instability of karyotypes and DNA fingerprinting during prolonged cultivation on agar slants, and 2) replacement of strains. We favour the first option, since instability of karyotypes has been observed in *C. neoformans* after mutagenesis.[53] Moreover, the rRNA genes occur on the largest chromosomes, and differences in the copy number of these genes may result in length polymorphism as well.[16] It has been suggested that the karyotypes of saprobic isolates of *C. neoformans* var. *gattii* vary less than the karyotypes of clinical isolates.[49] However, our finding of identical karyotypes and RAPD patterns for three clinical strains of *C. neoformans* var. *gattii* from India seems to contradict this observation.[54] Although the Asian clinical isolates differ slightly from the Australian *Eucalyptus* isolates, we do not consider the differences significant.

Different PCR typing strategies may result in detection of different amounts of genetic heterogeneity.[26,30,31,36] It is evident that RAPD analysis or PCR fingerprinting resolves clusters that, for instance, are not separated by serotyping. Immediate clinical application of the RAPD approach is still controversial, as

conflicting results have been obtained when multiple isolates from a single patient have been studied.[55,56] There are no generally accepted procedures for interpreting RAPD band patterns. Moreover, using different PCR protocols and/or different primers in different laboratories may result in different results.[42,57,58] It has been suggested that there may be a relationship between isolate characteristics and issues like body location or risk factors for the development of disseminated meningoencephalitis.[33] However, Varma et al. challenged these ideas after they encountered comparable genetic diversity in isolates from AIDS patients, non-AIDS patients, and the environment.[39] Our karyotype and RAPD type results are consistent with this latter observation. Therefore, it seems likely that infection is usually acquired by inhalation of saprobically living *C. neoformans.*

Our results suggest that RAPD type BA is dominant in AIDS patients, but this hypothesis may be biased by the sample containing a rather large number of isolates obtained from a local population of AIDS patients (Rotterdam strains). Discrimination of all Rotterdam isolates by using PFGE and RAPD analysis was not possible. The relatively homogenous karyotypes with concordant RAPD patterns, serotypes, and killer types of these isolates suggest that clonal expansion of the fungus occurred in this geographically restricted are, as has been revealed by linkage disequilibrium studies in other areas.[30] The observed local genetic homogeneity seems to contradict the results of other studies in which only a very small number of strains was found to be identical when PFGE and DNA fingerprinting were used.[33,35] Strains of *C. neoformans* var. *neoformans* from mastitic cows mainly were serotype D and RAPD type CD organisms, suggesting that there is some degree of genetic differentiation. However, more strains of veterinary origin need to be investigated to settle this issue. Our RAPD data suggest that there is geographical differentiation of *C. neoformans* var. *gattii.* When previously published DNA fingerprint patterns were compared with strain origins, a comparable geographic patterns was obtained.[39] There seems to be considerable genetic divergence between Australian and American populations, with the Asian and African populations somewhat intermediate. It has been suggested that this pattern may be correlated with the existence of some geographic and/or genetic substructure in *Eucalyptus* hosts.[59]

The following points summarize our results: 1) *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* differ in genetic makeup and may represent separate species; 2) no genetic differences were observed among clinical, veterinary, and saprobic isolates of either variety; 3) both varieties have various

numbers of chromosomes and various genome sizes, which probably reflect differences in ploidy and/or aneuploidy; 4) multiple isolates from a patient may exhibit minor karyotype differences; 5) karyotypes may not be stable after prolonged preservation on agar slants; 6) RAPD typing data suggest that there is a geographic substructure in *C. neoformans* var. *gattii*; 7) isolates from a local population of AIDS patients have relatively homogeneous PFGE and RAPD patterns; and 8) the combination of PFGE and RAPD analysis is useful in epidemiological research.

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Chapter 5

The use of lipid formulations of amphotericin B for systemic fungal infections.

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ABSTRACT

Despite its considerable toxicity, amphotericin B (AmB) remains the "golden standard" in the treatment of many systemic fungal infections. To reduce this toxicity, with the aim of increasing its therapeutic index, AmB can be encapsulated into liposomes or bound to lipid carriers. Following promising clinical results with investigational formulations, three industrial compounds are available at this moment: Abelcet (Amphotericin B Lipid Complex, ABLC), Amphocil (Amphotericin B Colloidal Dispersion) and AmBisome. These three formulations differ significantly in composition and pharmacokinetics. All three compounds share a considerable reduction of nephrotoxicity, but the number of acute reactions differ among these compounds, Amphocil showing the highest and AmBisome the lowest rate. Increased therapeutic indexes for all three formulations were shown only in some of the animal models for several fungal infections. Four recent clinical trials comparing these formulations with AmB demonstrated their clinical efficacy but failed to clearly show an increased therapeutic index. Therefore these compounds can be recommended in cases of intolerance to or failure on AmB therapy. The optimal therapeutic dosages have not been established, but dosages as low as 1 mg/kg should probably be avoided in the initial treatment of fulminant fungal infections, since efficacy may be inferior to equal dosages of conventional AmB.

INTRODUCTION

Owing to a lack of effective alternative antifungal agents, amphotericin B deoxycholate (AmB), despite its considerable toxicity, is still considered to be the treatment of choice for many invasive fungal infections.[1] Administration of the drug is associated with many adverse events including infusion-related side effects (fever, chills, hypotension), thrombophlebitis, nephrotoxicity, hypokalemia, hypomagnesemia, anemia and more rarely thrombocytopenia. Although the antifungal activity of AmB is dose-dependent, due to the above mentioned toxicity the maximum tolerated dosage is limited to 1.0-1.5 mg/kg/day. This phenomenon may contribute to the high failure rates in immunocompromised hosts.

To overcome the dose-limiting toxicity of AmB, other modalities of AmB have been developed in which this agent is encapsulated in liposomes or is bound to other lipid carriers. Liposomes are small spherical lipid structures with one or more phospholipid bilayers separated by an aqueous layer. Different molecules can be encapsulated both in the phospholipid bilayer and the aqueous layer. The lipophilic AmB is located within the lipid bilayers of the liposome. The liposomal formulations can be divided in large, multilamellar vesicles (MLVs) and small unilamellar vesicles (SUVs).[2] The size, bilayer rigidity and electrical charge are important factors for the disposition of liposomes after parenteral administration.[3] Large and negatively charged liposomes have a shorter circulation time in the bloodstream mainly due to a rapid uptake by the cells of the mononuclear phagocyte system (MPS). Smaller liposomes which are neutral or positively charged, have a longer circulation time. This time also increases when liposomes are more rigid, which can be accomplished by the incorporation of cholesterol in the lipid bilayer. Other, non liposomal, lipid formulations include carriers with the format of sheets or discs.

The reduction of toxicity of these lipid formulations may be the result of differences in affinity of AmB to the distinct lipid components.[4] The affinity of AmB to the ergosterol of the fungal membrane is the highest, that to the lipid carrier intermediate and that to the cholesterol of the human cell membrane the lowest. Therefore a selective transfer of AmB from the lipid carrier to the fungal membrane can be expected. Other mechanisms may contribute to a reduction of nephrotoxicity. The group of Lopez-Berestein demonstrated an increased binding of negatively charged liposomal AmB to high density lipoproteins which resulted in a decreased uptake of AmB in renal cells.[5] In contrast, AmB has a higher

binding to low density lipoproteins and is therefore taken up by low density protein receptors in the kidney cells.
Because the antifungal activity of most lipid formulations seems to be reduced to some extent, the question remains if the possibility to use higher non-toxic dosages might lead to an increased therapeutic index compared to AmB. This question was addressed in a wide variety of animal models, in which very high dosages of lipid formulations of AmB were used.

ANIMAL STUDIES

In most animal models both mycological and clinical efficacies were studied (Table 1).[6-26] These models involved a wide range of fungal infections: invasive candidiasis, aspergillosis, cryptococcosis, histoplasmosis, coccidioidomycosis and blastomycosis, in both immunocompetent and immunocompromised animals. Although an increased therapeutic index could be demonstrated for all lipid formulations, the results were not consistent (Table 1). An example of an increased therapeutic index was found in experiments performed in our institution in Rotterdam.[23] In a model of candidiasis, neutropenic mice were intravenously inoculated with *Candida* and treated for 5 days with various regimens of AmBisome, AmB or fluconazole; the numbers of colony forming units of yeasts in renal tissue were counted on different days of treatment. AmBisome was not effective in low dosages (0.3 mg/kg/day) in contrast to the same dosage of AmB. However, AmBisome showed superior activity when used in higher dosages (7 mg/kg/day) with eradication of the yeasts in most of the animals. We also have experience with a model of one-sided pulmonary aspergillosis in neutropenic rats (chapter 6). In this model we found that AmBisome and not AmB was able to reduce dissemination of infection in addition to reduction of the fungal burden at the initial infection site. This additional property was seen even at a relatively low dosage of 1 mg/kg/day of AmBisome. However, this 1 mg/kg/day regime did not result in a more prolonged survival.

Overall, in several animal models in which lipid based formulations were tested in an equal dose as AmB, the conventional formulation was more or at least as active as the lipid formulations. The only exception was the model for pulmonary aspergillosis, described by Francis et al, in which even relatively low dosages of liposomal amphotericin B (AmBisome) were more effective than AmB in reducing mortality rates.[19] When mycological efficacies were compared at

maximal doses, lipid formulations were generally more active than conventional AmB or otherwise at least as active as. However, when clinical efficacies (reduction of mortality) were compared, only few studies showed an increased therapeutic index. The various animal studies did not show consistency in relation to specific infections, specific doses and/or formulations (Table 1). Since in some studies increased efficacy was seen only when very high dosages were given, the relevance of these findings for clinical settings is questionable.

CLINICAL STUDIES WITH NON-COMMERCIAL COMPOUNDS

The first liposomal formulation of AmB to be tested in clinical settings was developed by Lopez-Berestein and co-workers. In 1985, two years after publication of the efficacy results in an animal model for candidiasis, they published the first clinical results of the use of this formulation in the treatment of deep fungal infections.[6,27] This formulation consisted of relatively large vesicles (MLV, diameter 1 - 8 μm) containing 5 mol% AmB (Table 2). The results they showed were promising in terms of safety and efficacy. After this publication it took another 4 years until they published the results of the treatment of a total of 46 patients.[28] Among these were 17 patients with chronic disseminated (hepatosplenic) candidiasis. The clinical response was especially good in this type of candidiasis with complete response in 13 out of 17 patients. These results can probably be explained by the characteristics of the liposome, which is very rapidly taken up by the MPS. In invasive aspergillosis, complete responses were documented in 12/29 cases. Although during the course of the study, dosages of up to 5.0 mg/kg/day were used, side effects were restricted to chills, mild fever and hypokalemia, substantial renal toxicity being absent. In a later paper however, a serious adverse event was reported: hypoxemia and depressed cardiac output during infusion in a 22-year old woman. The events were reversible but returned after later infusions.[29] Despite promising efficacy data of this formulation, it was not suitable for commercial development as it had to be prepared at the center shortly before therapy.

Another experimental liposomal formulation was developed at the Jules Bordet Institute in Brussels, Belgium.[30] This formulation consisted of SUVs (< 100 nm) which escaped uptake by the MPS, giving rise to high and sustained plasma levels (5- to 10-fold higher than after AmB) (Table 2). The tolerance of

Table 1. *Animal models comparing the efficacies of liposomal formulations of amphotericin B with that of amphotericin B deoxycholate.*

Liposomal amphotericin B; (dose (mg/kg))	Amphotericin B deoxycholate; (dose (mg/kg))	Infection / animal	Mycological efficacy[a,b]		Clinical efficacy[a,c]		Ref.
			equal dose	maximal dose	equal dose	maximal dose	
Amphocil							
1, 5, 10 iv	1 iv	pulmonary aspergillosis / rabbit[d]	nr	nr	Amb ≥ Lip[e]	Amb < Lip[e]	7
1, 5, 10 iv	1 iv	pulmonary aspergillosis / rabbit[d]	Amb > Lip	Amb = Lip	Amb = Lip	Amb = Lip	8
0.8 - 12.8 iv	0.2, 0.8 iv	disseminated cryptococcosis / mice	Amb = Lip	Amb < Lip	Amb = Lip	Amb = Lip	9
0.22 - 10 iv	0.22 - 1.3 iv	coccidioidomycosis / mice	Amb > Lip	Amb < Lip	Amb = Lip	Amb = Lip	10
Abelcet							
0.5, 1, 10 iv	0.5, 1 iv	candidiasis / mice	nr	nr	Amb = Lip	Amb < Lip	11
1, 10 iv	1 iv	cryptococcal meningitis / rabbit[f]	Amb = Lip	Amb < Lip	nr	nr	12
1, 10 iv	1 iv	candidiasis / rabbit	Amb > Lip	Amb < Lip	nr	nr	12
0.35 - 15 iv	0.3 - 7 iv/ip	coccidioidal meningitis / mice	Amb > Lip	Amb = Lip	Amb > Lip	Amb = Lip	13
0.22 - 20 iv	0.22 - 6.25 iv/ip	coccidioidomycosis / mice	Amb > Lip	Amb < Lip	Amb = Lip	Amb = Lip	14
0.05 - 3.2 iv	0.05 - 0.8 iv	candidiasis / mice[g]	Amb = Lip	Amb = Lip	Amb = Lip	Amb = Lip	15
0.2 - 12.8 iv	0.05 - 0.8 iv	disseminated aspergillosis / mice[f]	Amb > Lip	Amb < Lip	Amb > Lip	Amb < Lip	15
? - 12.8 iv	0.05 - 0.8 iv	disseminated cryptococcosis / mice	nr	Amb < Lip	Amb > Lip	Amb = Lip	15
? - 0.9 iv	nr - 0.8 iv	disseminated histoplasmosis / mice[h]	nr	nr	Amb = Lip	Amb = Lip	15
0.2 - 12.8 iv	0.2 - 6.3 iv/ip	blastomycosis / mice	Amb > Lip	Amb < Lip	Amb = Lip	Amb = Lip	16
AmBisome							
1, 10 iv	1 iv	pulmonary aspergillosis / rats[d]	Amb = Lip	Amb < Lip	Amb > Lip	Amb = Lip	17
0.3 - 7 iv	0.3 - 7 iv/ip	cryptococcal meningitis / mice	Amb > Lip	Amb < Lip	Amb > Lip	Amb = Lip	18

Table 1. *(continued)*

Liposomal amphotericin B; (dose (mg/kg))	Amphotericin B deoxycholate; (dose (mg/kg))	Infection / animal	Mycological efficacy[a,b]		Clinical efficacy[a,c]		Ref.
			equal dose	maximal dose	equal dose	maximal dose	
1 - 10 iv	1 iv	pulmonary aspergillosis / rabbit[d,f]	Amb < Lip	Amb < Lip	Amb < Lip	Amb < Lip	19
0.05 - 1.0 iv	0.05 - 1.0 iv	pulmonary coccidioidomycosis / mice	Amb = Lip	nr	nr	nr	20
8-30 iv	1-2 iv/ip	candidiasis / mice[d]	nr	Amb < Lip	nr	Amb = Lip	21
2 - 8 iv	1 - 3 iv	candidiasis / mice[f]	Amb > Lip	Amb = Lip	nr	nr	22
0.3, 7 iv	0.1 - 0.4 iv	candidiasis / mice[g]	Amb > Lip	Amb < Lip	Amb > Lip	Amb = Lip	23
1 - 20 iv	1 iv	blastomycosis / mice	Amb > Lip	Amb < Lip	Amb = Lip	Amb = Lip	24
0.75 - 10 iv	0.75 iv	candidiasis / mice	Amb = Lip	Amb < Lip	Amb = Lip	Amb = Lip	25
5 - 10 iv	1.5, 4.5 ip	disseminated cryptococcosis / mice	nr	Amb < Lip	nr	Amb = Lip	25
Prophylaxis AmBisome							
6.05 inhaled	6.73 inhaled	pulmonary aspergillossis / mice[d]	Amb < Lip	nr	Amb = Lip	nr	26

Ref. = reference, iv = intravenously, ip = intraperitoneally, nr = not recorded, Amb = Amphotericin B deoxycholate, Lip = liposomal formulation.

[a]Symbols used to describe efficacy data: =: equally effective as, >: more effective than, <: less effective than.

[b]Mycological efficacy was determined by differences in the number of Colony Forming Units.

[c]Clinical efficacy was determined by differences in survival, with the exception of reference 7.

[d]Animals were rendered granulocytopenic.

[e]Clinical efficacy was determined by differences in lesions seen on Ultrafast Computerized Tomographic Scans.

[f]Animals received corticosteroids.

[g]A part of the animals were rendered granulocytopenic.

[h]A part of the animals received corticosteroids.

Table 2. *Characteristics of different amphotericin B formulations (after ref. 28)*

Formulation	Lipid composition (molar ratio)	AmB Mol %	Format	Diameter (μm)	Cmax[a]	AUC[b]
Fungizone (conventional AmB)	deoxycholate	34%	micels	<0.4	=	=
Lopez-Berestein[b] (AmB-liposome)	DMPC/DMPG (7:3)	5%	MLVs / sheets	1-8	lower	smaller
Jules Bordet[b] (AmB-liposome)	EPC/CHOL/SA (4:3:1)	<5%	SUVs	<0.10	higher	larger
Abelcet (AmB-Lipid Complex)	DMPC/DMPG (7:3)	35-50%	sheets	1.6-11	lower	smaller
Amphocil (AmB-Colloidal Dispersion)	cholesterylsulfate (1:1)	50%	discs	0.12 x 0.004	lower	equal to
AmBisome (AmB-liposome)	HPC/CHOL/DSPG (2:1:0.8)	10%	SUVs	0.08	higher	larger

AmB=amphotericin B, AUC=area under the concentration-time curve, CHOL=cholesterol, Cmax=maximum serumconcentration, DMPC=dimirystoyl-phosphatidylcholine, DMPG=dimirystoylphosphatidylglycerol, DSPG=distearoylphosphatidylglycerol, EPC=egg-phosphatidylcholine, HPC=hydrogenated phosphatidylcholine, MLV=multi lamellar vesicles, SA=stearylamine, SUV=small unilamellar vesicles.

[a]Related to equal dosages of conventional AmB.

[b]Not industrially produced.

this formulation was very good: no renal toxicity was observed. Despite this, the formulation has never been commercially available.

Meanwhile, pharmaceutical companies have developed three lipid based alternatives: Abelcet® (Amphotericin B Lipid Complex, ABLC), Amphocil® (Amphotericin B Colloidal Dispersion, ABCD) and AmBisome®. It is important to stress that these compounds differ significantly in structural properties, pharmacokinetics and tolerance (Table 2).[2] Therefore, each lipid formulation of AmB should be evaluated separately.

ABELCET (AMPHOTERICIN B LIPID COMPLEX, ABLC)

Although under a different name, Abelcet (The Liposome Co., Princeton, N.J., USA) was the first commercial available lipid formulation of AmB. Recently it has been approved by the FDA for the use in the treatment of systemic aspergillosis. The product is related to the original large liposome of Lopez-Berestein and composed of the same two phosphatidylesters. By increasing the ratio of AmB above 25 mol%, the liposomes aggregated and sheets with relatively large size (10 μm) were formed.[31] Due to this large size, these sheets are rapidly removed from the blood by the liver and spleen, which results in low serumlevels (AUC 0.4 x that of AmB). It is important to note that all reported serumlevels of lipid formulations are total concentrations of carrier-bound, lipoprotein-bound and unbound fractions of AmB. Preliminary data from noncomparative studies with Abelcet showed good results in patients with mycoses who were unresponsive to or intolerant of AmB.[32] The clinical response rates were 78% for candidiasis and 60% for aspergillosis, respectively. Recently preliminary results of a randomized trial comparing Abelcet 5 mg/kg and AmB 1 mg/kg in the treatment of hematogenous and invasive candidiasis in neutropenic and nonneutropenic patients, were published.[33] The clinical response rates were comparable between the two regimes (Abelcet 63%, AmB 68%), but renal toxicity was significantly reduced in Abelcet-treated patients (doubling of serumcreatinine in 28% versus 47%). However, there was no difference as to other side effects (chills, nausea) between the two regimes. An increased therapeutic index could not be demonstrated with the dosages used in this study. Definite results of a comparative trial between 3 dose regimes of Abelcet and one regime of conventional AmB for cryptococcal meningitis in AIDS patients, were published by Sharkey et al.[34] Patients were randomly assigned to receive Abelcet 1.2, 2.5, 5.0 mg/kg/day or

AmB 0.7 mg/kg/ day for 2 weeks followed by 4 weeks of Abelcet 2.5, 5.0, 5.0 or AmB 1.2 mg/kg thrice weekly respectively. The total number of adverse events, infusion related events (chills, rigors, nausea etc.), and the occurrence of hypokalemia were high when compared to previous experience, but similar for all treatment groups. The mean change from baseline values of serum creatinine significantly favored Abelcet regimes. No significant differences in clinical, mycological, or overall responses could be observed, although the authors suggest that this was partly due to the low number of patients and dissimilarities in the groups produced by randomization.

AMPHOCIL (AMPHOTERICIN B COLLOIDAL DISPERSION, ABCD)

Amphocil (Liposome Technology, Inc., Menlo Park, CA, USA) is a colloidal dispersion of a stable complex of AmB with cholesterylsulfate in a 1:1 proportion.[35] The format is that of flat discs. The peak serumlevels of Amphocil are lower than those for AmB, but due to a longer half-life, the AUC is about the same. In phase I and II studies, infusion of relatively low dosages of 0.25 to 1.5 mg/kg resulted in a rather high rate of the well known side effects of AmB: nausea 53%, headache 40%, thrombophlebitis 33%, vomiting 20% and chills 20%.[36] Therefore, it has been recommended to use infusion rates of 1 mg/kg/h.[37] Up to now efficacy data are scanty. A study in 24 patients with coccidioidomycosis treated with 1 mg/kg, showed modest efficacy with failure or relapse rates of 54% after 9 weeks follow-up and a rather high rate of adverse events.[38] At this moment, phase II studies using mean daily doses of 3.1 mg/kg, and a phase III study for pulmonary aspergillosis using 4 mg/kg/day, are performed.[39]

AMBISOME

AmBisome (NeXstar Pharmaceuticals, San Dimas, CA, USA) is a liposomal formulation now registered in 18 countries worldwide. This liposome is a SUV which can build up serum levels higher than that after administration of AmB (Table 2). We found peak serum levels up to 80 μg/ml in AIDS patients treated with 4 mg/kg/day (personal observations, unpublished). In a compassionate use study published in 1991, data of 137 episodes of AmBisome therapy were analyzed including 64 cases of mycologically proven invasive infections.[40] The clinical response rates in this heterogenous population of patients were encouraging: 61% response in invasive aspergillosis and 84% in invasive candidiasis.

The frequency of acute reactions was low (1% chills) even without the use of additional drugs as corticosteroids.[41] A rise in serum creatinine was seen in 9% and a decrease of serum potassium in 28% of the patients. In addition, a number of patients showed a rise of serum transaminases. However, from these noncomparative data the clinical significance of the latter finding remains unclear. Recently there have been two reports of minor and major anaphylactic reactions following the initial administration of AmBisome but not AmB.[42,43] Although the frequency of these adverse events is low, close surveillance during the first administration is recommended.

Another noncomparative study in 116 neutropenic patients included 57 patients with documented (n=21) or suspected (n=36) aspergillosis.[44] The response rate was 62% in the documented cases and 53% in patients with suspected aspergillosis. Dosages up to 5 mg/kg/day were used with a low number of acute side effects (chills, rigors or nausea in 5%) and no significant nephrotoxicity. However the median dosage was 133 mg/day which equals about 2 mg/kg/day which is relatively low. The median leukocyte count of the responders was significantly higher than that for nonresponders who did not show any bone marrow recovery.

AmBisome also showed good results in a noncomparative study for the treatment of cryptococcal meningitis in 23 AIDS-patients with a rather serious form of infection as indicated by a median intracranial pressure of 30 cm water.[45] A favorable response was seen in 78%, a mycological response in 67% and a fatal outcome in 13%. One of the striking findings was a median CSF culture conversion time of only 11 days, which compares favorably with AmB plus flucytosin (16 days) and fluconazole 200-400 mg (60 days).[45,47]

Only recently the preliminary results of two comparative and randomized studies in patients with fever of unknown origin (FUO) were presented.[48,49] The first report described the results in 203 neutropenic paediatric patients with FUO not responding to 96 hours of systemic antibiotic treatment. These patients were randomized to receive AmBisome 1 or 3 mg/kg or AmB 1 mg/kg. Overall the number of clinical adverse events was higher in the AmB group. Complete resolution of symptoms was observed in 75.8%, 63.4% and 52.5% of AmBisome 1, AmBisome 3 mg/kg and AmB groups respectively. The second report described the results in a group of adult patients with FUO not responding to 96 h of systemic antibiotic treatment (n=134) or confirmed mycosis (n=52). The number of reported adverse events was significantly lower in the AmBisome treated

patients. Resolution of symptoms did not significantly differ among the three groups of patients with FUO. In patients with confirmed mycosis, clinical cure rates were 70% (12/19), 50% (8/17) and 35% (6/17) for AmBisome 1, AmBisome 3 mg/kg and AmB groups respectively; no information on the causal fungus was given. So, in the setting of FUO and early fungal infection, doses as low 1 mg/kg AmBisome may be effective. This finding might be in accordance with a more effective reduction of dissemination after AmBisome, as shown in our animal study.[17]

AMB-INTRALIPID EMULSION

In addition to the commercial products, the efforts of French investigators, who produced an emulsion at the bedside by mixing intralipid 20% with AmB, have to be mentioned.[50] A dosage of 1 mg/kg AmB in this emulsion showed less toxicity than equivalent dosage of AmB. In another study a mean dosage of 1.2 mg/kg was effective in 13 out of 14 episodes of candidemia in neutropenic patients.[51] A dosage of 1.5 mg/kg appeared to be less successful in the treatment of cryptococcal meningitis: only 50% (5/10) of the patients showed a conversion of the CSF culture.[52] Furthermore, because instability of this formulation and the formation of precipitations in this mixture have been demonstrated, its use has been discouraged.[53]

DISCUSSION

Abelcet, Amphocil and AmBisome share the advantage of a low nephrotoxicity. However, the number of acute reactions is not equally diminished in these formulations. The rate of acute reactions seems to be minimal for AmBisome and highest for Amphocil. This diminished toxicity may lead to an increased therapeutic index. Such an increased therapeutic index has only be demonstrated in a number of, but not all animal models. Extrapolation of these results to the clinical setting is very difficult. The first four comparative studies using different lipid formulations and AmB showed promising results as to toxicity. In both comparative trials with Abelcet, one in candidemia and the other in cryptococcal meningitis, no increased therapeutic index could be demonstrated.[33,34] Although AmBisome seemed to be superior to AmB in the management of FUO in neutropenic patients, both studies failed to show an increased therapeutic index for the higher

AmBisome dosage (3mg/kg).[48,49] There is still a need for more randomized studies in well defined populations with proven invasive mycosis, comparing the compounds with standard therapy with AmB.[54] We have analysed the results of two comparative trials with AmBisome: one in neutropenic patients with confirmed mycosis or highly probable aspergillosis (chapter 7) and one in patients with AIDS and cryptococcal meningitis (chapter 9). In the meantime the issue remains of what dosages should be used. Most animal studies suggest that lipid formulations exert their optimal efficacy at dosages higher than the maximum dosage (1-1.5 mg/kg) of AmB. In addition when lipid formulations were given in equivalent dosages, they showed efficacy rates inferior to that of AmB. It has recently been shown for AmBisome in a study with liver transplant recipients and in two FUO studies that these dosages may be useful in prophylactic settings or in early fungal infection.[48,49,55] Until definite data of comparative trials are available we would still not recommend the use of dosages as low as 1 mg/kg/day for the initial treatment of acute fulminant fungal infections.

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Chapter 6

AmBisome reduces dissemination of infection as compared to amphotericin B deoxycholate in a rat-model of pulmonary aspergillosis.

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SUMMARY

The efficacy of AmBisome, a liposomal formulation of amphotericin B, was compared to that of Fungizone (amphotericin B desoxycholate), in a rat model of one-sided aspergillosis. Repeated administration of cyclophosphamide resulted in persistent, severe, granulocytopenia. The left lung was inoculated with a conidial suspension of *Aspergillus fumigatus*, thus establishing a one-sided infection. Antifungal treatment was started 40 hr after fungal inoculation, at which time mycelial disease could be confirmed by histological examination. Both Fungizone 1 mg/kg and AmBisome 10 mg/kg resulted in increased survival in terms of delayed as well as reduced mortality. Quantitative cultures of lung tissue showed that only AmBisome 10 mg/kg resulted in reduction of the number of colony forming units in the inoculated left lung. Compared to Fungizone, both AmBisome 1 mg/kg and AmBisome 10 mg/kg prevented dissemination from the infected left lung to the right lung significantly. In addition both AmBisome regimes reduced hepatosplenic dissemination as well. AmBisome 10 mg/kg fully prevented hepatosplenic dissemination. In conclusion, when compared to Fungizone, in this model AmBisome is more effective in reducing dissemination of one-sided pulmonary aspergillosis, even in relatively low doses. These doses may have a place in prophylactic settings.

INTRODUCTION

Aspergillus species are opportunistic fungi, giving rise to pulmonary and other invasive infections in immunocompromised patients.[1-5] The number of invasive aspergillus infections is steadily increasing.[5-7] This is due to growing numbers of susceptible patients, especially those with prolonged granulocytopenia after aggressive chemotherapy. Despite considerable toxicity amphotericin B is still the most effective agent to treat these infections. However the efficacy of this drug is rather disappointing in persistently granulocytopenic patients in whom mortality rates up to 100% have been observed.[4]

Encapsulated into liposomes amphotericin B has considerably diminished toxicity, and can be administered in much higher doses.[8-12] The administration of higher doses may result in improved efficacy, as the antifungal activity of amphotericin B is concentration dependent. We compared the efficacy of amphotericin B deoxycholate (Fungizone®) to that of liposomal amphotericin B (AmBisome®) in an animal model of pulmonary aspergillosis that closely mimics human disease; one-sided onset of infection of the lung, persistent granulocytopenia and start of treatment at a time when hyphal growth is firmly established.

MATERIALS AND METHODS

Animals. Female R strain albino rats (specified pathogen free; 18 to 25 weeks old; weight 185 to 235 g; bred at REPGO-TNO, Rijswijk, The Netherlands) were used for all experiments. Both the control group and the three treatment groups consisted of fifteen animals each. Animals received a normal, pathogen free diet and water ad libitum.

Induction of granulocytopenia and supportive care. According to a slight modification of the method described by Roosendaal et al., persistent granulocytopenia was induced by one dose of cyclophosphamide 90 mg/kg intraperitoneally 5 days before fungal inoculation followed by repeated doses of cyclophosphamide 60 mg/kg at 1 day before and 3, 7 and 11 days after inoculation.[13] The doses of cyclophosphamide were adjusted for body weight changes during the experiment. Prior to the experiments with amphotericin B this scheme was tested in a group of 6 animals. Quantitation of blood leukocytes was done in blood samples obtained by orbital puncture under light CO_2 anesthesia. Total leukocyte counts were determined from diluting these samples in Türk solution in a hemacytometer. Total numbers of granulocytes were calculated after differential

counts of leukocytes in cytospin preparations of buffy coats.

To prevent bacterial superinfections strict hygienic care was applied and animals received ciprofloxacin (660 mg/L) and polymyxin B (100 mg/L) in their drinking water during the whole experiment. Starting one day before inoculation daily amoxicillin (40 mg/kg/day/i.m.) was added to this regime for the remainder of the experiment.

***Aspergillus fumigatus* strain.** A strain of *Aspergillus fumigatus* isolated from an immunocompromised patient with invasive pulmonary aspergillosis was used. This strain was stored under oil on Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, United Kingdom) slopes during the study. At least once every two months, the strain was passed through a rat to maintain it's virulence. The MIC and MFC of the *A. fumigatus* strain for amphotericin B, determined by the methods of Schmitt *et al.* (1988), were 0.4 and 0.8 mg/L, respectively. There were no differences in MIC and MFC values if a high inoculum ($1x10^5$ conidia per ml) versus a low inoculum ($5x10^3$ conidia per ml) was used.

Experimental lung infection. *A. fumigatus* was cultured at 37°C for 96 hr on SDA until a dense mycelium with abundant conidia had formed. These conidia were harvested in 10 ml sterile saline. Conidia were removed from the thallus by rubbing the colony-surface gently with a glass pipette. This resulted in a conidial suspension without hyphal elements (microscopically checked). The suspension was washed twice in sterile phosphate buffered saline (PBS). Subsequently, conidia were resuspended in sterile PBS (pH 7.4) and counted in a hemacytometer. In addition, the viability of the conidia was determined by culturing 10-fold serial dilutions on SDA.

Infection of the left lung was established according to the methods described by Bakker-Woudenberg *et al.*[14] Briefly, under general anesthesia the left main bronchus was intubated. A cannula was passed through the tube and the left lobe of the lung was inoculated with 0.02 ml of the conidial suspension. This resulted in a left-sided pneumonia. Sixty minutes before this procedure animals received a prophylactic i.m. dose of gentamicin (6 mg/kg) and an extra i.m. dose of amoxicillin (40 mg/kg).

Antifungal treatment. Amphotericin B deoxycholate (Fungizone®, Bristol Myers-Squibb, The Netherlands) and liposomal amphotericin B (AmBisome®, Vestar, San Dimas, CA) were reconstituted as prescribed and further diluted in 5% glucose in water. Daily doses were administered intravenously into the tail vein over a period of at least 30 sec, in a volume of 1.0 ml or less. Animals received one of the following regimens: 1 mg/kg Fungizone, 1 mg/kg AmBisome

or 10 mg/kg AmBisome (these dosages were calculated a priori and not adjusted for weight-changes during the experiment). A comparison dose of 10 mg/kg of Fungizone was not investigated for the LD_{50} of of Fungizone in rats is 1.6 mg/kg.[10] Treatment was started 40 hr after fungal inoculation, at which time mycelial disease could be confirmed by histological examination of lung tissue, and was continued for 10 consecutive days.

Toxicity. Our study was not designed to investigate in detail the toxicity associated with the treatment regimens. However, to check for the occurrence of gross toxicity, renal and hepatic functions were monitored. Abnormalities of renal functions were detected by measuring serum creatinine and BUN (blood urea nitrogen) after ten days of administration of Fungizone 1 mg/kg and AmBisome 10 mg/kg. Abnormalities of hepatic function were detected by measuring serum aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT).

Therapeutic endpoints. Several endpoints were used to evaluate the efficacy of antifungal treatment. Animals were checked twice every day and mortality was recorded for 12 days after fungal inoculation. In addition, surviving animals were sacrificed 24 hr after the last dose of antifungal agent to determine the extent of fungal disease present at that time. From all animals that died or were sacrificed the left and right lung, liver and spleen were aseptically removed and homogenized in 20 ml distilled water in a homogenizer (The VirTis Co. Inc., Gardiner, NY, USA) for 45 sec at 10,000 rpm. Liver and spleen of each rat were homogenized together. Serial tenfold dilutions of the homogenates were prepared in distilled water and 0.2 ml samples of each dilution as well as 2 ml samples of the undiluted homogenates were spread on to SDA plates which were incubated at 37°C for 48 hr. Colony forming units (cfu's) were counted after 24 and 48 hr of incubation. The remainders of the organ homogenates were poured into SDA plates and incubated at 37°C for 48 hr, in order to count very low numbers of cfu's or to prove sterility of the organs.

Statistical analysis. For statistical analysis of differences in survival curves Wilcoxon two-tailed test of life tables was used. This test examines the decrease in survival with time as well as the final percentage of survival. Differences in proportions of animals with dissemination to the right lung, liver and/or spleen were examined by Fischer's exact test. Differences in mean log cfu were examined by Mann-Whitney test. *P*-values of less than 0.05 were considered significant in these analyses.

RESULTS

Granulocytopenia. The dosing scheme of cyclophosphamide used was sufficient to keep animals persistently granulocytopenic (granulocytes < $0.1x10^9$/L) from the onset of infection (time zero) and continuing throughout the whole experiment. The number of total leukocytes as well as granulocytes is shown in Figure 1. Differential counts showed that less than 10% of the leukocytes consisted of granulocytes.

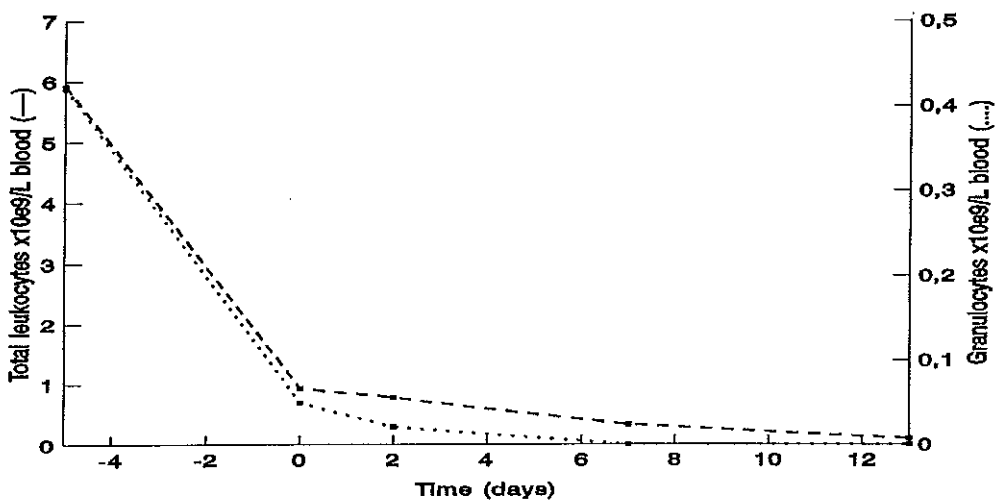

Figure 1. Number of total leukocytes and neutrophilic granulocytes determined in the blood of rats (n=5) treated intraperitoneally with cyclophosphamide 90 mg/kg at day -5, and repeated doses of 60 mg/kg on days -1, 3, 7 and 11.

Experimental infection. Inoculation of relatively low numbers of conidia ($1x10^2$ or $1x10^3$) resulted in survival of all or 80%, respectively of the animals for at least twelve days after fungal inoculation. Inoculation of higher numbers of conidia ($1x10^4$ or $5x10^4$) resulted in 100% mortality of animals, the first animals dying on day four after fungal inoculation (Fig. 2). In all experiments on efficacy of antifungal treatment an inoculum of $1x10^4$ conidia was used.

The size of the inoculum also influenced the time at which histologically proven mycelial disease was observed. Inocula of $1x10^2$, $1x10^4$ and $5x10^4$ resulted in mycelial disease at $\geq$ 48 hr, 40 hr and 30 hr after inoculation, respectively. Histological examination revealed abundant septate branching hyphae

invading blood-vessels and lung tissue. Macroscopically, often gross hemorrhagic infarctions were seen, even at a time when infectious lesions were still small.

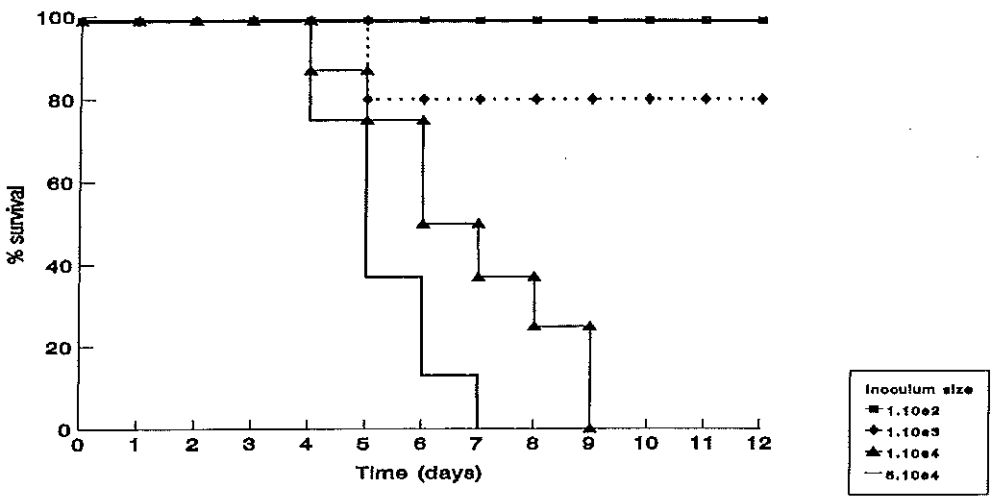

Figure 2. *Survival of granulocytopenic rats after inoculation of the left lung with various numbers of A. fumigatus conidia: $1x10^2$ or $1x10^3$ (n = 5), $1x10^4$ or $5x10^4$ (n = 8).*

If left untreated, inoculation with $1x10^4$ conidia, initially resulted in a left-sided invasive pulmonary infection. After several days the infection disseminated and *A. fumigatus* could be detected in the right lung, the liver and the spleen.

Efficacies of AmBisome vs. Fungizone in granulocytopenic rats with aspergillosis. Following fungal inoculation all untreated animals died (Fig. 3). Mortality was observed starting from day 5 after inoculation onwards; at day 9 all animals had died. AmBisome 1 mg/kg did not influence survival significantly. Both Fungizone 1 mg/kg and AmBisome 10 mg/kg resulted in increased survival (P = 0.006 and P = 0.027, respectively) in terms of delayed as well as reduced mortality. Only 2/15 (13%) animals treated with Fungizone 1 mg/kg and 4/15 (27%) animals treated with AmBisome 10 mg/kg survived the study period of twelve days. Survival patterns between the animals treated with these regimens did not differ significantly (P = 0.75).

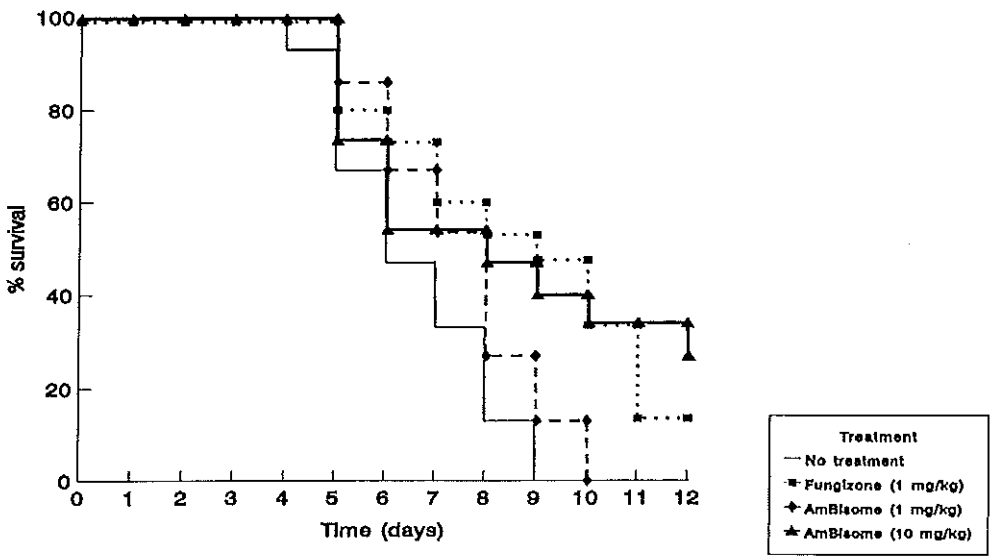

Figure 3. *Effect of AmBisome versus Fungizone on survival of granulocytopenic rats inoculated with 1x10[4] A. fumigatus conidia. Groups of 15 animals each received Fungizone 1 mg/kg, AmBisome 1 mg/kg or AmBisome 10 mg/kg for 10 consecutive days. Treatment was started 40 hr after fungal inoculation, at what time mycelial growth was firmly established.*

The results of the quantitative cultures of the internal organs are shown in Table 1. The number of cfu's found in the left lung was not significantly reduced following treatment with Fungizone 1 mg/kg or AmBisome (1 mg/kg). In contrast, treatment with AmBisome 10 mg/kg resulted in a significant reduction of the number of cfu's in the left lung (P = 0.003).

Fungizone 1 mg/kg had no effect on the dissemination of the infection to the right lung, the number of animals with infected right lungs as well as the number of cfu's found in these lungs being the same as in the untreated animals. Dissemination to the right lung after treatment with AmBisome both at 1 mg/kg and 10 mg/kg did not differ significantly with untreated animals, however, both regimes reduced dissemination to the right lungs when compared to treatment with Fungizone (P = 0.014). In addition, the numbers of cfu's found in the right lung when dissemination had occurred, were significantly smaller than those

Table 1. *Effect of AmBisome versus Fungizone on the dissemination of A. fumigatus in granulocytopenic rats.*

Treatment	Left lung		Right lung		Liver and Spleen	
	% pos.	median log_{10} cfu	% pos.	median log_{10} cfu	% pos.	median log_{10} cfu
None	100	3.7 (1.8-4.3)	80	2.5 (1.6-2.5)	66	1.9 (0.7-2.5)
Fungizone 1 mg/kg	100	3.5 (2.7-3.9)	93	2.0 (0-2.5)	27	1.4 (0.6-1.7)
AmBisome 1 mg/kg	100	3.3 (2.6-3.7)	47[a]	0.9 (0.7-2.0)[b]	7[c]	0.5 (0-0.9)
AmBisome 10 mg/kg	100	3.1 (1.9-3.9)[b]	47[a]	1.1 (0-2.3)[b]	0[c]	0

Note: All animals were challenged with $1x10^4$ *A. fumigatus* conidia in the left lung and followed for up to 12 days.

[a] $P < 0.02$ compared to Fungizone treated animals (not significant compared to untreated animals), Fischer's exact test.

[b] $P < 0.01$ compared to untreated animals, Mann-Whitney test.

[c] $P < 0.01$ compared to untreated animals, Fischer's exact test.

found in untreated animals ($P = 0.0008$ and $P = 0.0012$, respectively).

All treatment regimens reduced dissemination of the infection to the liver and the spleen. However, only with AmBisome both at 1 mg/kg and 10 mg/kg, these differences reached statistical significance ($P = 0.0017$ and $P = 0.0002$, respectively). AmBisome 10 mg/kg completely prevented dissemination of the infection to these organs. During these experiments we did not study dissemination to the brains of the animals. However, in the brains of none of the untreated animals (n = 10) viable fungal elements were found.

Toxicity. Serum creatinine, BUN, ALAT and ASAT levels after ten days of administration of the antifungal regimens used did not show any significant increases (data not shown).

DISCUSSION

Due to various reasons the number of invasive infections caused by *A. fumigatus* is still increasing and probably will increase further in the coming years. Antifungal agents that can be relied upon with regards to prophylaxis and therapy are not yet available. Currently, amphotericin B deoxycholate (Fungizone) is the drug of choice for treating invasive pulmonary aspergillosis. Newly developed antifun-

gals should, therefore, be compared to Fungizone to evaluate their efficacy. Because administration of Fungizone is associated with a number of side effects, the development of new antifungals with the same efficacy but less toxicity would be a major step forward. Liposomal formulations of amphotericin B are proven to be considerably less toxic than amphotericin B deoxycholate thus allowing much higher doses (up to ten-fold).[8-10,12] AmBisome is a commercially available liposomal formulation (small unilamellar vesicles) containing amphotericin B.[10]

Invasive pulmonary aspergillosis is an opportunistic infection usually diagnosed in patients who have been treated with corticosteroids and/or chemotherapeutic agents. In various animal models of aspergillosis corticosteroids are administered.[15-20] However, corticosteroids have been reported to cause combined toxicity with amphotericin B.[15,21] To avoid the possible influence of this combined toxicity on survival rates of animals, in the present study cyclophosphamide was used to obtain persistent, severe, granulocytopenia. Because with this regimen the viability of pulmonary macrophages is not affected, we examined the histology of the left lung at different times after fungal inoculation to ascertain that conidia had germinated at the time antifungal treatment was started. It was found that the time to germination depended on the inoculum size. Antifungal treatment was started when invasive lesions already had developed, which is comparable to the clinical situation; most other published models ignore this pathophysiological state. It was observed that the size of macroscopic lesions did not correlate with the numbers of viable *A. fumigatus* cultured from those lungs. Francis *et al.* reported similar observations.[22] The invasion of blood vessels by *Aspergillus spp.* and the consequent extensive hemorrhagic infarction of lung tissue might help explain these findings. The left lung of a rat consists of a single lobe, so hyphal invasion of the vessels of this lung might cause alteration of the macroscopic aspect of the entire lung at a relatively early stage.

In man pulmonary aspergillosis is often initially detected in one of both lungs. In the rat, by intubating the left main bronchus we were able to inoculate only the left lung with conidia of *A. fumigatus*, which allowed us to study progression of the infection to the right lung, a phenomenon often seen in clinical situations. Furthermore this study allows one to study drug pharmacokinetics in infected lung tissue versus uninfected lung tissue in the same animal. By intubating the left main bronchus it is possible to inoculate rats in a standardized manner, as demonstrated by the relatively small range of days at which untreated animals die. The same observations were made by Schmitt *et al.* who inoculated intratracheally.[17]

The efficacy of antifungal agents in invasive aspergillosis is very much influenced by the duration of deep granulocytopenia.[4] In man persistent, severe granulocytopenia is associated with mortality rates up to 100% despite treatment with amphotericin B. In the present animal model, rats were granulocytopenic during the whole experiment to mimic human conditions. The mortality rate was dependent on the size of the inoculum. This inoculum-dependent-mortality was described before, and might be explained by the ability of pulmonary macrophages to clear a certain amount of viable conidia from the lung.[20] By increasing the number of conidia administered it was possible to increase the number of animals dying as well as to delay the onset of mortality. The inoculum which resulted in 100% mortality with the first animals dying at day 5, was chosen to study the efficacy of antifungal treatment. The onset of mortality is of importance in clinical settings. A delay in the progression of infection which can be accomplished by antifungal treatment should offer the patient additional time to repopulate his/her bone marrow. From clinical experience it is known, that bone marrow recovery is a crucial factor predicting a favorable outcome. In the present model all untreated animals died early. AmBisome 1 mg/kg did not significantly influence survival, in contrast, both Fungizone 1 mg/kg and AmBisome 10 mg/kg did increase survival by almost two days; both treatment regimes resulted in the survival of some animals (13% and 27%, respectively). These percentages are relatively low when compared to those reported by Francis *et al.*[22] We believe this to be largely due to differences between the respective experimental model; for example antifungal therapy was started earlier than in our model. The importance of these differences are underscored by the fact that not all of their control animals died during the study period. The observations in the present study resemble those found in clinical practice where, despite antifungal treatment, survival percentages are less than 10-20% in patients with persistent granulocytopenia.

Clear differences were found in the number of organs which were infected at the time animals died or were sacrificed. AmBisome in both the low and the high dosage prevented dissemination from the infected left lung to the right lung in more than 50% of the animals, where Fungizone did not prevent such dissemination to the other lung. These findings were rather surprising, as van Etten *et al.*, found, in uninfected mice, that after administering of AmBisome, lung tissue levels were lower as compared to levels found after equal doses of Fungizone.[23] It is possible that AmBisome is distributed more directly to the fungal elements and surrounds these elements, or, if liposomes are taken up by macrophages,

this might alter local concentration of the drug possibly resulting in higher concentrations at places of infection.[24] Furthermore, the relatively prolonged blood circulation of AmBisome resulting in increased serum concentrations compared to serum concentrations obtained after administration of Fungizone, may help to prevent hematological fungal dissemination.[10] These results suggest a role for relatively low doses of AmBisome in a prophylactic setting.

The significant reduction of viable *A. fumigatus* after treatment with 10 mg/kg AmBisome in both the infected left lung and the organs involved in dissemination may indicate that this regime is able to keep fungal lesions limited. In clinical settings reduction of the number and size of the lesions may improve success rates in cases of bone marrow recovery.

Both treatment regimes of AmBisome significantly reduced hepatosplenic dissemination. However, only the higher dose of AmBisome was able to fully prevent this dissemination. This may be due to the fact that high levels of AmBisome accumulate in these organs.[22] In the present study mortality did not seem to be directly correlated to the degree of dissemination of infection. This might be due to the relatively acute and progressive type of pneumonia which was induced. Respiratory insufficiency resulting from vascular involvement of the left lung, rather than dissemination in itself, may thus be the direct cause of death in the present model. This hypothesis, however, needs to be confirmed.

We conclude that in this model AmBisome is more effective than Fungizone in reducing dissemination of pulmonary aspergillosis. Relatively low doses of AmBisome may have a place in prophylactic settings. The optimal treatment doses of AmBisome need to be further defined.

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Chapter 7

AmBisome compared with amphotericin B deoxycholate in the treatment of neutropenia-associated invasive fungal infections.

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SUMMARY

Background: It has been suggested that a better outcome of neutropenia-associated invasive fungal infections can be achieved when high doses of lipid formulations of AmB are used.

Methods: A randomized multicenter study comparing AmBisome 5 mg/kg/-day to AmB 1 mg/kg/day in the treatment of these infections.

Results: Of 106 tentatively included patients, 66 were definitely enrolled and analyzed for efficacy: 9 documented fungemia, 17 otherwise documented invasive mould infections and 40 suspected pulmonary aspergillosis. After completion of medication, in the AmBisome group (n=32) 14 patients had a complete response, 7 a partial response and 11 a failure as compared to 6, 13 and 15 patients (n=34) treated with AmB (P=0.09); P=0.03 for complete responders. A favorable trend for AmBisome was found at day 14, in patients with documented infections and in patients with pulmonary aspergillosis (P=0.05 and P=0.096 respectively). Mortality rates were less in patients treated with AmBisome (adjusted for malignancy status, P=0.03). More patients on AmB had a >100% increase of their baseline serum creatinine (P<0.001).

Conclusion: In neutropenic patients with documented or suspected invasive fungal infections AmBisome 5 mg/kg/day is superior to AmB 1 mg/kg/day with respect to efficacy and safety.

INTRODUCTION

Invasive fungal infections in hemato-oncologic patients with neutropenia continue to have high morbidity and mortality rates [1-5]. Some reports have suggested a better outcome if higher doses (up to 1.5 mg/kg/day) of amphotericin B deoxycholate (AmB) are used [1, 6]. However, such regimens are associated with high toxicity rates. Lipid formulations of AmB, which have shown to be less nephrotoxic have recently been developed [7-9]. AmBisome (NeXstar Pharmaceuticals, Inc., San Dimas, California, USA) is the only truly liposomal formulation of AmB and has demonstrated promising results in both animal models of invasive fungal infections and in clinical studies in neutropenic patients with fever of unknown origin (FUO) [8, 10]. A more rapid mycological response to AmBisome as compared to AmB was seen in a study including HIV-infected patients with cryptococcal meningitis [11].
Here we report the results of an open label, randomized comparative multi center trial of AmBisome 5 mg/kg/day versus AmB 1 mg/kg/day in the treatment of neutropenia-associated invasive fungal infections.

METHODS

Study Population

Hospitalized patients $\geq$ 16 years of age with untreated documented or highly suspected invasive fungal infection were eligible for tentative enrollment following informed consent. Patients had to be severely neutropenic (neutrophilic granulocytes $< 0.5 \times 10^9$/L) or to present within an interval of 14 days following recovery from severe neutropenia. Patients could be tentatively enrolled either on the basis of 1) positive fungal culture or histology from blood or deep tissue specimen, or 2) positive culture or microscopy showing fungal hyphae from sputum or from broncho-alveolar lavage (BAL) fluid together with chest X-ray abnormalities compatible with pulmonary infection, or 3) the presence of fever not responding to broad-spectrum antibiotics plus chest X-ray abnormalities suggestive of pulmonary aspergillosis as judged by the pulmonologist. In every patient suspected of pulmonary aspergillosis, a BAL was required before entry to confirm fungal infection and exclude other etiology. During the first 1.5 year of the study only patients with fungal infection that was documented within seven days after enrollment were definitely enrolled. After this 1.5 year, patients with suspected pulmonary aspergillosis were also eligible for definite enrollment provided no

other etiology was established. The protocol required that tentatively enrolled patients that did not fulfill criteria for definite enrollment were replaced by new patients. All replaced patients were included in the toxicity evaluation.

At entry the following characteristics were recorded: medical history, underlying malignancy, previous anti-cancer therapy and malignancy disease status at entry (partial remission, complete remission, stable disease or progressive disease). Patients with progressive disease either received their first induction therapy for acute leukemia, had relapsing progressive leukemia or had refractory hematological malignancy.

The study protocol was reviewed and approved by the ethics committees of all participating Dutch and, according to French regulation, by the ethics committee of one French center.

Antifungal Therapy

After tentative enrollment, patients were randomized 1:1 to receive either AmBisome 5 mg/kg/day i.v. or AmB 1 mg/kg/day i.v.. Randomization was performed centrally by one Dutch and one French center stratified by participating institution. After two weeks of full dose, the AmBisome and AmB doses were reduced to 3 mg/kg/day and 0.7 mg/kg/day respectively, provided their neutrophils raised over $0.5x10^9$/L, otherwise full doses were continued for as long as neutrophils remained below that count.

AmBisome was administered immediately at full dose infused over 45 min; no central intravenous catheter was required. The dose of AmB was escalated to a full dose over a 24 h period and was infused over six h; the use of a central intravenous catheter was recommended. Sodium supplementation prior to AmB infusion to prevent nephrotoxicity and medication to prevent acute reactions was allowed.

Dose adjustments were required when serum creatinine rose from normal to $\geq$ 300 % or from an already elevated baseline value to $\geq$ 200 %. Study medication was then to be discontinued for 2 days and subsequently to be reinstituted at half dose, when creatinine levels had fallen again. If a rise in serum creatinine did not recur, doses were again escalated to full; if serum creatinine remained at the above mentioned high levels after five days of reduced doses, the study medication was discontinued.

Clinical Evaluation and Outcome Assessment

Efficacy. During the first four weeks on study, patients were evaluated daily for

vital signs, temperature, specific signs of fungal infection and side effects. Physical examination was performed frequently and, in case of pulmonary infection, chest X-rays at least once a week. During follow-up patients were examined and a chest X-ray was made every two weeks. Chest X-rays of patients with documented or suspected pulmonary aspergillosis were blindly reevaluated by a pulmonologist with respect to localization, extension and nature of lesions at the outset, changes during treatment and time to improvement.

The following criteria were used for clinical evaluation: 1) Complete response: normalization of all pre-treatment signs and symptoms together with, if applicable, progressive improvement of chest X-rays. 2) Partial response: decrease of pretreatment signs and symptoms and a stable or improved chest X-ray. 3) Failure: unchanged or progressive pretreatment signs and symptoms. 4) Relapse: recurrence of any sign or symptom of fungal infection during follow-up after an initial response. After discontinuation of study drugs patients were followed for four weeks to document relapses. Mortality was evaluated during treatment and follow-up.

Toxicity. Blood urea nitrogen (BUN), serum creatinine and potassium were determined daily and additional chemistry (including liver enzymes) and hematologic parameters weekly.

Discontinuation of therapy. Study drug treatment was prematurely discontinued in case of a sustained rise in serum creatinine levels, other serious adverse events judged to be study drug related by the treating physician or if requested by the patient or physician.

Serum Concentrations of Amphotericin B

Trough and peak serum samples were obtained after 3 and 8 days of therapy. Levels of amphotericin B were all measured by High Performance Liquid Chromatography at the Erasmus University Medical Center Rotterdam, as previously described [12].

Statistical Analysis

The study was primarily powered to show superiority of AmBisome over AmB in achieving a more rapid clinical response (complete and partial). Assuming a difference in response rates of 40 % after 14 days between AmBisome and AmB, 30 definite enrolled patients in each arm should suffice (two-sided p=0.05, power=90%). Cumulative percentages of patients with clinical response were calculated according to the method of Kaplan and Meier. Comparison of these curves

was done with the log-rank test. The relation between response rates and neutropenic status was determined using Cox-regression with time-dependent variables [13]. Graded response outcomes or percentages were compared the Mann-Whitney *U* test or Fisher's exact test, respectively. Approximate normally distributed variables were compared by Student's t-test. Comparison of mortality, taking account of malignancy status was done using exact logistic regression. The change from baseline of logarithmically transformed creatinine values was compared using repeated measurements analysis of variance (RmANOVA) [14]. A p-value ≤ 0.05 was considered significant.

All analyses of clinical and mycological efficacy data of definitely included patients were performed on an intention-to-treat basis and went on till all antifungal treatment was discontinued. For analysis of toxicity, patients were evaluated up to the time of definite discontinuation of the study drug.

RESULTS

Study Population

From January 1992 through January 1996, 106 patients with documented or suspected invasive fungal infection were tentatively enrolled in the study. All were evaluated for toxicity. A total of 40 patients were ineligible for definite enrollment for the following reasons: during the first 1.5 year, 18 patients were withdrawn because no fungi had been cultured from pretreatment materials within 7 days; after protocol revision, 15 patients were excluded because pretreatment nonfungal causes were found to explain symptoms and 4 patients because invasive candidiasis could not be documented. Two patients were ineligible because of previous treatment with itraconazole and lack of pretreatment cultures respectively. One patient was tentatively enrolled when *A. fumigatus* was cultured from his sputum but judged ineligible for definite enrollment when it became apparent that this was due to mass laboratory contamination (>100 culture media were contaminated). Of the 66 definite enrolled patients, 32 had been assigned to AmBisome and 34 to AmB. Demographic characteristic, clinical parameters, duration of neutropenia until enrollment, sort and status of underlying malignancy, the number of bone marrow transplantations and, baseline hematologic laboratory values did not differ significantly between the groups (Table 1). Baseline levels of creatinine, BUN, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase and bilirubin did not differ significantly either. Sites of infection among the 66 patients were as followed: 9 documented fungemia (*Can-*

Table 1. *Demographic characteristics, clinical parameters, underlying malignancies and, baseline laboratory of 66 neutropenic patients with documented or suspected invasive fungal infection.*

Characteristic	Amphotericin B deoxycholate (n=34)		AmBisome (n=32)	
Age - yr				
Median	48		52.5	
Range	20-68		18-74	
Sex - number of patients (%)				
Male	10	(29)	6	(19)
Female	24	(71)	26	(81)
WHO status - number of patients (%)				
Fully active	7	(21)	5	(16)
Restricted activity	6	(18)	8	(25)
Fatigue, full self-care	8	(24)	6	(19)
Limited self-care	10	(29)	12	(32)
Completely disabled	3	(9)	1	(3)
Malignancy - number of patients (%)				
Acute nonlymphocytic leukemia/ myelodysplastic syndromes	20	(59)	18	(56)
Acute lymphocytic Leukemia	2	(6)	6	(19)
Chronic Leukemias	3	(9)	2	(6)
Other	9	(26)	6	(19)
Previous BMT[a] - number of patients (%)	5	(15)	4	(13)
Previous SCT[a] - number of patients (%)	1	(3)	0	(0)
Malignancy status - number of patients (%)				
Complete remission	9	(26)	6	(19)
Partial remission	4	(12)	3	(9)
Stable disease	4	(12)	4	(13)
Progressive disease	16	(47)	19	(59)
Unknown	1	(3)	0	(0)
Duration of neutropenia prior to enrollment - days				
Median	15		19	
Range	5-94		3-113	
Neutropenic at enrollment - number of patients (%)	30	(88)	30	(94)
Hemoglobulin (g/dL)				
Median	6.15		6.45	
Range	4.9-12.0		5.1-9.5	
Leukocytes ($x10^9$/L)				
Median	0.4		0.3	
Range	0.1-19.8		0.0-22.4	
Neutrophils ($x10^9$/L)				
Median	0.05		0.03	
Range	0.0-17.8		0.0-6.8	
Creatinine (μmol/L)				
Median	70		83	
Range	30-187		21-195	

[a]BMT = bone marrow transplantation, SCT = stem cell transplantation.

dida spp. n=7, *Cryptococcus neoformans* n=1 *Fusarium* spp. n=1), 17 otherwise documented invasive mould infections (15 pulmonary infection, 1 aspergillosis of a vertebra, 1 sinusitis caused by *Absidia* species) and 40 suspected pulmonary aspergillosis (Table 2).

The median duration of neutropenia after enrollment was 5.5 days (range 0-98) for patients assigned to AmBisome versus 6.5 days (range 0-36) for patients assigned to AmB (P=0.30). Six patients had recovered within 14 days from neutropenia, 4 assigned to AmB (for 1, 6, 11 and 14 days) and 2 assigned to AmBisome (1 and 12 days).

Table 2. *Causal agents in 66 neutropenic patients treated for documented or suspected invasive fungal infections according to assigned antifungal therapy.*

Fungal Infection	Amphotericin B deoxycholate (n=34[a])	AmBisome (n=32[a])
Documented:		
Aspergillus fumigatus/spp.	4	7
Mould not further determined	4	1
Other moulds[b]	2	0
Candida albicans	1	2
Candida non-albicans	1	3
Yeast not further determined	0	1
Other yeasts[c]	1	1
Suspected:		
Pulmonary aspergillosis	22	18

[a]Two patients (one in each arm) had more then one fungal pathogen isolated.
[b]*Fusarium spp* and *Absidia spp.*
[c]*Saccharomyces cerevisiae* and *Cryptococcus neoformans.*

Efficacy

Outcome at day 14 and at completion of therapy. The median duration of treatment was 14.5 (range 2-149) days for patients on AmBisome versus 16.5 (range 4-76) for patients on AmB.

Table 3. *Outcome at day 14, at completion of therapy and mortality of 66 neutropenic patients treated for documented or suspected invasive fungal infections with either amphotericin B or AmBisome.*

	Amphotericin B deoxycholate (N=34)				AmBisome (N=32)				
	Complete response	Partial response	Failure	NE[a]	Complete response	Partial response	Failure	NE[a]	*P*-value
Response at day 14									
All patients	2	6	25	1	5	10	15	2	*0.03*
- with documented infection	0	2	10	0	4	2	6	2	*0.05*
- with pulmonary aspergillosis	2	6	20	1	3	10	12	1	*0.096*
Response at completion									
All patients	6	13	15	0	14	7	11	0	*0.09*[b]
- with progressive malignancy	1	4	11	0	6	3	10	0	*0.19*[b]
- with documented infection	2	3	7	0	9	0	5	0	*0.07*[b]
- with pulmonary aspergillosis	6	11	12	0	11	7	8	0	*0.16*[b]

Mortality (number of patients)	Amphotericin B deoxycholate	AmBisome	*P*-value
All patients	13/34	7/32	*0.19*[c]
- with progressive malignancy	12/16	7/19	*0.04*
- with non-progressive malignancy	1/18	0/13	*1.00*
- with documented infection	6/12	4/14	*0.42*
- with pulmonary aspergillosis	11/29	5/26	*0.15*

[a]3 patients were not evaluable at day 14, because no chest X-ray was performed (n=2) or because no signs and symptoms were noted.

[b]*P*=0.03, *P*=0.096, *P*=0.02 and *P*=0.14 respectively for proportion of patients with complete response.

[c]Adjusted for malignancy status: *P*=0.03.patients with suspected or documented pulmonary aspergillosis, 11 (42%) patients on AmBisome and 6 (21%)

As shown in Table 3, at 14 days, 5 (17%) patients assigned to AmBisome had a complete response, 10 (33%) a partial response and 15 (50%) had a failure versus 2 (6%), 6 (18%) and 25 (76%) patients assigned to AmB (P=0.03), overall response (complete or partial) rates being 15/30 versus 8/33 (P=0.04). For patients with documented fungal infection overall response rates were 6/12 (50%) in AmBisome patients versus 2/10 (20%) in AmB patients (P=0.19); for patients with suspected or documented pulmonary aspergillosis overall response rates were 13/25 (52%) in AmBisome versus 8/28 (29%) in AmB patients (P=0.10).

At completion of therapy, in the AmBisome arm 14 (44%) patients had a complete response versus 6 (18%) in the AmB arm (P=0.03). Among patients with documented infection, 9 (64%) AmBisome patients (5 aspergillosis, 1 mould infection and 3 yeast infections) and 2 (17%) AmB patients (both with moulds not further determined) had a complete response (P=0.02). Within the AmB arm, the patient with *Absidia* sinusitis died, the patient with fusaremia improved. Amongpatients on AmB had a complete response (P=0.14).

The overall response rates were mainly determined by the malignancy status of the patients being 40% in patients with progressive disease versus 83% in patients in remission or with stable disease (P=0.001). When the time to response was evaluated, patients who recovered from their neutropenia had a 4.9-fold (95% confidence interval 2.1-11.0) increased response rate as compared to patients who remained neutropenic (P<0.001). No differences existed in the proportion of patients recovered from neutropenia at day 14 between the treatment arms: 17/32 versus 20/34. The differences in response rates between patients recovering and not recovering from neutropenia were the same for AmBisome and AmB. The results of patients who had recovered from neutropenia within 14 days prior to enrollment (n=6), were not different than the overall outcome.

Mortality. Overall mortality appeared to be determined considerably by the malignancy status of patients. Mortality rates were 54% among patients with progressive malignancy and 3% in those who were in remission or had stable disease (P<0.001). Taking account of the malignancy status using exact logistic regression, the overall mortality rate in patients assigned to AmBisome was significantly lower than in the AmB group: 7/32 (22%) versus 13/34 (38%) (P=0.03). In the AmBisome arm 5/7 and in the AmB arm 10/13 patients were considered to have died due or at least partly due to fungal infection. Among patients with progressive malignancy mortality rates were 7/19 (37%) in the

AmBisome group versus 12/16 (75%) in the AmB group (P=0.04).

Mycological response. In patients with documented fungal infection, at completion of therapy, 7/10 patients assigned to AmBisome showed fungal eradication (4/5 moulds and 3/5 yeasts) versus 2/8 (2/6 moulds and 0/2 yeasts) patients assigned to AmB (P=0.15); 8 patients were not evaluable for mycological response.

Chest X-rays. Chest X-rays of 47/55 patients with suspected or documented pulmonary aspergillosis were available for the blinded retrospective analysis. For the remaining eight patients the judgement of the local radiologist was accepted. The number of patients with focal lesions was comparable between the treatment arms: 10/26 versus 11/29. A significantly higher percentage of patients with focal lesions showed complete or partial resolution of X-ray abnormalities, 81% versus 52% in patients with diffuse lesions (P=0.04). Clinical response percentages in these subgroups were 67% versus 48% (P=0.24). No differences were found either in resolution of chest X-ray abnormalities nor in clinical response rates between patients with bilateral and unilateral lesions. Analysis showed that the number of patients who had improving chest X-rays was not significantly different between the two treatment arms, 17/26 (65%) for AmBisome versus 16/29 (55%) for AmB (P=0.58). Median time to radiological improvement was 3 weeks in the AmBisome group versus 5 weeks in the AmB group (P=0.71).

Toxicity

The number of patients who received concomitant nephrotoxic medication was high both in patients given AmBisome and AmB: aminoglycosides (23/52 versus 23/54), glycopeptides (47/52 versus 40/54) and diuretics (7/52 versus 11/54). Duration of antifungal treatment and cumulative doses are shown in Table 4. Both treatment regimens were generally well tolerated (Table 4). RmANOVA showed that 14 days after start of treatment the mean change from baseline serum creatinine was 86% (±9%) in the AmB and 1.4% (±5%) in the AmBisome group (P<0.001). Significantly more patients treated with AmB had a more than 100% increase of their baseline serum creatinine; 22/54 (40%) versus 6/51 (12%) (P<0.001). In 18 patients treated with AmB and in two patients treated with AmBisome, medication was temporarily discontinued or lowered in dose due to increase of serum creatinine (P<0.001). The number of patients with major changes in biochemical parameters are shown in Table 4. Three patients treated with AmBisome and 7 treated with AmB, had to stop therapy because of toxicity (P=0.31). One AmBisome treated patient had to stop because of hepatotoxicity,

Table 4. Duration of therapy, cumulative dose and the number of patients with adverse events during treatment with amphotericin B or AmBisome for neutropenia associated invasive fungal infections.

Characteristic	Amphotericin B deoxycholate (n=54)	AmBisome (n=52)
Duration of therapy (days)		
Median (range)	13 (3-76)	14 (1-149)
Cumulative dose (mg)		
Median (range)	850 (216-3,836)	3,865 (360-27,000)
Adverse event		
Clinical		
Fever/chills	12[a]	5[a]
Nausea	1	0
Exanthema	3	4
Chest pain/tachypnoea	2	3
Other acute reactions[b]	3	5
Detected in laboratory		
Fall in Hemoglobin > 2.0 g/dL	3	2
Hypokalemia (≤ 2.5 meq/L)	11	10
Creatinine > 2 times baseline	22[c]	6[c]
Aminotransferase > 5 times ULN[d]	7	7
Alkaline Phosphatase > 5 times ULN[d]	5	3
Bilirubin > 5 times ULN[d]	8	9
None of these adverse events reported	24	23

[a] P=0.11 (Fisher's exact test).
[b] AmB: hypotension, tickling cough and wheezing. AmBisome: hypotension, organic psychosyndrome, headache, oedema of the legs and acute allergic reaction.
[c] P<0.001 (both Fisher's exact test)
[d] ULN = Upper Limit of Normal.

one because infusion-related retrosternal pain and one because of an anaphylactic reaction after infusion, which recurred after switching to AmB. The reason to stop AmB treatment was renal toxicity in all cases.

Serum Concentrations of Amphotericin B

Median serum trough levels (1.80 μg/ml, range 0.34-45.80) and peak levels (19.10 μg/ml, range 5.98-80.00) of amphotericin B in patients treated with

AmBisome, were higher than those in patients treated with AmB (trough, 0.67 μg/ml, range 0.2-1.32 and peak 1.43 μg/ml, range 0.40-2.89; P=0.022 for trough and P< 0.0001 for peak levels). Median serum trough or peak levels at day 3 and 8 did not differ.

DISCUSSION

Amphotericin B deoxycholate (AmB) is the drug of choice for the (empirical) treatment of invasive fungal infections in neutropenic patients, however, its efficacy in this setting is disappointing [1-5]. Only fluconazole showed to be more effective than AmB in a subgroup of neutropenic patients with candidiasis in one larger study including only patients with candidal infections [1, 15, 16]. However, the lack of activity against moulds prohibits the empirical use of fluconazole in a setting where mould infections have to be covered such as the febrile neutropenic patient with pulmonary infiltrates. We report an antifungal agent with superior clinical efficacy compared to AmB in the treatment of neutropenia associated invasive fungal (yeast and mould) infections. AmBisome showed a significantly higher response rate within 14 days and more patients had a complete response at termination of therapy. A complete response to antifungal therapy offers better opportunities for continuing treatment of the underlying malignancy, whereas fungal infections that only partially respond to therapy are more likely to relapse during renewed immunosuppressive therapy. However, patients with a complete response might also relapse from a cryptogenic fungal focus [17]. Although the majority of infections could not be documented, we feel that this does not devaluates the results of this study because in a clinical setting these are the exact patients that are started on antifungal therapy. Furthermore, patients with other documented (microbiological) diagnoses in which normally antifungal treatment should also be discontinued, were not eligible for definite enrollment; these patients were not allowed to receive further free study medication and could therefore not be included in the intention to treat analysis.

The favorable trend in response rates to AmBisome seen in patients with documented infection may be influenced by the two arms being not completely comparable for fungal causes. However, both in patients with fungemia and in patients with documented aspergillosis or other mould infection, clinical responses among patients treated with AmBisome were better. Patients with progressive malignancy, including patients who received first induction chemotherapy,

responded far less to antifungal therapy. In patients with progressive malignancy mortality was twice as high among patients on AmB as compared to patients on AmBisome. Therefore, an analysis taking account of the malignancy status was performed. This showed that adjusted mortality among all patients was significantly lower in those treated with AmBisome.

In patients with documented or suspected pulmonary aspergillosis a trend was found in favor of AmBisome. The results of these patients showed less differences between the treatment arms which may be due to the fact that in the subgroup of patients with suspected aspergillosis, patients without a fungal infection might have been included. Compared to CT-chest, chest X-rays are known to be extremely heterogeneous and less sensitive, particularly in early cases of invasive aspergillosis. With the current knowledge that CT-chest is the most sensitive and therefore preferred radiological method to diagnose invasive aspergillosis, future studies should include regular CT's instead of chest X-rays which were requested in our study [18]. Earlier diagnosis of invasive aspergillosis by using CT may also greatly influence outcome of therapy.

Resolution of neutropenia is the most important factor influencing outcome of invasive aspergillosis [1, 18]. In this study patients recovering from neutropenia had a five-fold increased response rate, nevertheless, we chose to include patients who recovered from neutropenia within the preceding 14 days because they acquired the infection probably during neutropenia and therefore belong to the same population at risk. Differences of efficacy could not be explained from differences in time to recovery of neutrophils between both arms which did not exist. Remarkable was the fact that in 7 patients (4 on AmBisome and 3 on AmB) clinical symptoms had cleared before the neutrophils were restored.

Our study is the first clinical trial to show superior clinical efficacy of a lipid formulation of AmB over the parent drug in the treatment of invasive fungal infections. As yet, three lipid products of AmB have been marketed in Europe or the US: Abelcet (Amphotericin B Lipid Complex), Amphocil (Amphotericin B Colloidal Dispersion) and AmBisome [7-9]. The major advantage of these products is reduction of toxicity allowing the administration of higher doses which should result in an increased therapeutic index. Indeed, this has been reported in several animal models [8]. The three formulations differ significantly in pharmacological characteristics and, although they are all less nephrotoxic than AmB, it has been shown that they differ in the rate of other side-effects, especially acute infusion-related reactions [8, 19, 20]. Therefore, these products need to be compared separately to the parent compound to evaluate their efficacy and safety.

The results found in the present study using AmBisome, thus, should not be extrapolated to the other formulations. Until now, mostly compassionate use results had been published concerning the lipid products [21-25]. Data of comparative studies with Amphocil are not available. Two comparative studies with Abelcet and two with AmBisome have been published [10, 11, 26, 27]. No differences in efficacy between Abelcet and AmB could be demonstrated in a small study of AIDS-associated cryptococcal meningitis nor in a larger study including a diversity of patients (of which less than 15% was neutropenic) with invasive candidiasis [26, 27]. A comparative study with AmBisome in neutropenic patients with FUO showed equal and possibly superior results for AmBisome [10]. Furthermore, AmBisome showed a better mycological response in patients with AIDS-associated cryptococcal meningitis, although, clinical efficacy was not different [11]. The present study shows that AmBisome has an increased therapeutic index in the treatment of invasive fungal infections in neutropenic patients, because it combines less toxicity with higher clinical efficacy.

The optimal dose of AmBisome remains to be determined. From the results of studies in patients with FUO, it was concluded that lower doses may be effective, although it is very difficult to assess antifungal efficacy in studies with these particular group of patients [10]. Results of most animal studies indicate that the highest doses give the best responses [8]. However, in an animal model of pulmonary aspergillosis, it was shown that lower doses of AmBisome were able to prevent dissemination of infection [28]. It could therefore be hypothesised that doses as low as 1 mg/kg may well be useful in a prophylactic setting, such as febrile neutropenia. We propose that future clinical studies should address the question whether higher doses (e.g $\geq$10 mg/kg/day) of AmBisome can further increase its efficacy in more advanced infections. Toxicity data from the present study clearly indicate that maximal tolerated doses in patients exceed 5 mg/kg/-day.

In this study the response rates of patients with progressive malignant disease to antifungal therapy were much lower. This lower response rate was apparently not caused by a longer duration of neutropenia, for this duration was the same in patients with and without progressive malignancy. It is possible that the functioning of neutrophils in patients with progressive malignancy is less than when the malignancy is in remission. Because the results of antifungal therapy between these two subgroups is completely different, we recommend that in future comparative studies patients are stratified according to their status of malignancy at entry. Differences in the proportion of patients who are in remissi-

on can easily confound the observed differences in outcome between treatment arms. If this information is not available, it is pointless to compare the results of different studies. The status of malignancy might also be taken into account when antifungal therapy is selected. Therapy with liposomal amphotericin B may be especially indicated for patients with progressive malignancies and possibly for patients with other factors predictive of a poor outcome such as bone-marrow recipients.

We were surprised to see that serum levels of amphotericin B after administration of AmBisome 5 mg/kg/day were significantly lower than after the administration of AmBisome 4 mg/kg/day in a study we performed simultaneously in patients with AIDS-associated cryptococcal meningitis [11]. This difference in pharmacokinetics profile points to a higher volume of distribution of AmBisome in the neutropenic patient compared to the HIV-infected patient. Similar observations have been reported for other drugs in these patient groups [29, 30]. The relevance of serum levels as to efficacy, however, is still unclear.

We conclude that in neutropenic patients with documented or suspected invasive fungal infections high dose (≥5 mg/kg) AmBisome was superior to standard AmB with respect to efficacy and safety. AmBisome could be the therapy of choice in the treatment of invasive fungal infections especially in patients with progressive malignancy.

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Chapter 8

Cryptococcal meningitis in HIV-infected patients: CSF opening pressure as prognostic factor.

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ABSTRACT

Objective. To study the value of prognostic factors for outcome of cryptococcal meningitis in HIV-infected patients.

Patients and methods. A retrospective study was performed in sixteen consecutive HIV-infected patients who experienced a first episode of cryptococcal meningitis and were admitted to the Academic Hospital Rotterdam-Dijkzigt.

Results. Sixteen patients were included. All presented with headache. Signs of meningeal irritation were noted in only 5/16 (31%). All patients were diagnosed by culturing *Cryptococcus neoformans* from the cerebrospinal fluid (CSF) or the brain.

Six patients had a complicated infection course; three of them died. All patients with a complicated infection course had a CSF opening pressure greater than 30 cm H_2O at the time of diagnosis. This proved to be the only factor predictive of a complicated course of cryptococcal meningitis in this group of patients.

Conclusion. A CSF opening pressure > 30 cm H_2O at the time of diagnosis may be predictive of a complicated course of cryptococcal meningitis in HIV-infected patients. The value of CSF opening pressure as a prognostic factor should be evaluated in prospective trials.

INTRODUCTION

Cryptococcosis is the most common systemic fungal infection in HIV-infected patients with a reported incidence of 5 to 10 %.[1,2] In about 50% of these patients cryptococcosis is the AIDS-indicator disease.[3] Cryptococcal meningitis is the most frequent manifestation. The mortality of this infection is high (up to 22%).[4] In non-AIDS patients several factors are considered to be of prognostic value.[5] In AIDS patients, however, the course of the infection is more indolent and the value of prognostic factors seems to be different. The cerebrospinal fluid (CSF) opening pressure is one of the possible valuable parameters in HIV-infected patients.[5] We retrospectively studied the course of cryptococcal meningitis in 16 consecutive patients with cryptococcal meningitis treated in our institution and related it to the CSF opening pressure at admission and other possible prognostic factors.

PATIENTS AND METHODS

Between January 1985 and October 1993, 491 HIV infected patients consulted our institution; during this study period 208 patients developed AIDS. Only patients with a primary episode of culture proven cryptococcal meningitis were included. Their medical records were analyzed for the following: demographic data, CD4+ cell counts at the time of diagnosis, presenting signs and symptoms, serum cryptococcal antigen (CRAG) titer, results of CSF examination (opening pressure, protein, glucose, white blood cell count, CRAG titer, India ink stained preparations and cultures), results of extraneural cultures and computed tomographic (CT) scans of the brain.

A complicated course was defined as the occurrence of seizures, irreversible cranial nerve palsies or death during initial treatment, or a relapse of the infection, despite maintenance therapy, within a period of 10 weeks after diagnosis.

The prognostic value of the following factors was evaluated: age, duration of complaints, CD4+ cell count, mental status, serum sodium and CRAG titer, CSF opening pressure, CSF CRAG titer, CSF white blood cell count (WBC), India ink preparation results and extraneural culture results. The statistical significance of the prognostic factors was determined by Fisher's exact test.

RESULTS

Sixteen of 208 HIV-infected patients experienced a primary episode of cryptococ-

cal meningitis (7.7%). In 8/16 patients this was the AIDS-indicator disease. Signs and symptoms at presentation are shown in Table 1.

Table 1. *Signs and symptoms at presentation.*

Sign/symptom	number of patients (percentage)	
Headache	16	(100)
Temperature > 38°C	12	(75)
Malaise, nausea, vomiting	11	(69)
Altered mental status	5	(31)
Stiff neck	5	(31)
Photophobia	2	(13)

A lumbar puncture was performed in 15 patients (one patient died before CSF examination was performed). Encapsulated yeasts were seen in India ink preparations, and *Cryptococcus neoformans* was cultured from the CSF of all these patients. In one patient, *C. neoformans* was cultured (post-mortem) from the brain. Serum CRAG titers were positive in 13/13 patients (in three patients titers were not determined). The CSF opening pressure was raised in 10/12 patients; the CSF CRAG titer was positive in 12/12 patients; WBC counts, glucose and protein values varied considerable. Abnormalities were found on CT's of the brain of 7/15 patients, however, the observed abnormalities were not specific for cryptococcal meningitis. In five patients *C. neoformans* was cultured from materials other than CSF (urine; urine; broncho-alveolar lavage fluid and blood; brain, heart, kidney, spleen, liver and lymph-nodes; blood).

Patients with positive India ink preparations immediately received therapy. Initial treatment consisted of fluconazole (400 mg/day) in three, amphotericin B (0.3-0.7 mg/kg/day) with flucytosine (150 mg/kg/day) in eight, amphotericin B (0.7 mg/kg/day) alone in three and liposomal amphotericin B (4 mg/kg/day) (AmBisome) in two patients. All surviving patients were discharged with fluconazole maintenance therapy.

Three patients (19%) died; two of them within 48 hours after admission to the hospital. Five patients experienced seizures; three of them also developed irreversible cranial nerve palsy (optical and facial nerve; optical, facial and abducent nerve; optical and acoustic nerve, respectively). One patient relapsed

Table 2.[a] *Possible prognostic factors, and clinical course of cryptococcal meningitis in 16 HIV-infected patients.*

Patient characteristics				Serum[b]			CSF[b]				initial treatment[c]	clinical course
age (years)	duration complaint (days)	mental status	extraneural cultures	sodium (mmol/L)	CRAG titer	CD4 ($x10^9$/L)	OP (cm H_2O)	India ink	CRAG titer	WBC count ($/mm^3$)		
30	35	normal	ND	141	2,048	0.05	ND	+	128	13	AmB/5FC	uncomplicated
38	28	altered	+	122	65,536	0.01	ND	+	ND	ND	AmB/5FC	uncomplicated
50	14	altered	ND	124	ND	0.02	25	+	256	3	AmB/5FC	uncomplicated
36	11	normal	+	137	16	0.02	15	+	ND	30	AmB/5FC	uncomplicated
53	7	normal	ND	137	131,072	0.03	30	+	1,048,576	36	AmB/5FC	uncomplicated
34	14	altered	-	139	1,024	0.15	ND	+	8,196	53	AmB/5FC	uncomplicated
44	7	normal	-	138	128	0.08	15	+	8	4	AmB	uncomplicated
41	14	normal	-	137	1,024	0.05	26	+	1,024	94	L-AmB	uncomplicated
46	6	normal	ND	137	ND	0.05	21	+	2,048	53	AmB	uncomplicated
36	9	normal	-	128	512	0.04	25	+	4,096	13	AmB	uncomplicated
34	28	altered	ND	142	ND	ND	39	+	512	138	fluconazole	seizure, died day 1
49	4	altered	+	ND	2,048	0.10	80	+	1,024	8	fluconazole	seizure, CNP, died day 31
28	4	normal	+	136	32,728	0.02	75	+	128	2	fluconazole	seizure, relapse
45	35	normal	-	123	4,096	0.19	55	+	ND	86	AmB/5FC	seizure, CNP
48	13	normal	+	ND	16,384	ND	ND	ND	ND	ND	AmB/5FC	died day 1
31	14	normal	-	139	8,192	0.02	32	+	32,768	ND	L-AmB	seizure, CNP

[a]CSF = Cerebrospinal Fluid; CRAG = Cryptococcal Antigen; OP = Opening Pressure; WBC = White Blood Cell; CNP = cranial nerve palsy.

[b]ND = not done; + = positive; - = negative.

[c]AmB = amphotericin B; 5 FC = flucytosine; L-AmB = liposomal amphotericin B.

three weeks after discharge from the hospital. He was treated successfully with amphotericin B. Ten patients had an uncomplicated course of cryptococcal meningitis.

Several reported risk factors for poor outcome were investigated in relation to the course of the infection (Table 2). None of these factors was significantly correlated to a complicated course of infection in this group of patients. In addition, duration of complaints, CD4 counts and serum CRAG titer were not predictive of a complicated course. The only factor we found to be clearly predictive of a complicated course of cryptococcal meningitis was the CSF opening pressure. Patients with a complicated course of their infection had CSF opening pressures which were significantly higher (range 32-80 cm H_2O, mean 56.2, SD 21.2) than those of patients with an uncomplicated course (range 15-30 cm H_2O, mean 22.4, SD 5.7) ($P<0.05$).

DISCUSSION

The timely diagnosis of cryptococcal meningitis is a major problem in HIV infected patients, because clinical presentation is often subtle and nonspecific.[1,2] Most patients present with headache, fever and malaise. Symptoms of meningeal involvement are frequently absent. Despite difficulties with retrospective analysis of presenting symptoms it seemed clear at least 63% of our patients had no signs of meningeal irritation. Overall signs and symptoms corresponded well with those known from literature.[1,2,6]

A tentative diagnosis can be made by determining the serum CRAG titer and by the visualization of encapsulated yeasts in India ink preparations; both tests had a sensitivity of 100% in this study. Especially the serum CRAG titer is a simple and very sensitive test which should be performed in HIV-infected patients with headache of unknown origin.[6] The golden standard for a definite diagnosis, however, remains the isolation of *C. neoformans* from the CSF.

It is important to start antifungal therapy as soon as possible. Optimal therapeutic schemes for HIV-infected patients are not determined yet. There is evidence that severe cases of cryptococcal meningitis should initially be treated with amphotericin B with or without flucytosine and not with azoles.[7-9] Compared to fluconazole, amphotericin B (0.7 mg/kg/day) with flucytosine had superior efficacy in one study.[7] Saag et al. used relatively low doses of amphotericin B (at least 0.3 mg/kg/day), but even these sterilized the CSF earlier than fluconazole did.[8]

They concluded that fluconazole should only be used as initial therapy in patients who are at low risk for treatment failure. Patients at low risk for treatment failure are those with relative mild infections.

In order to decide whether initially to treat with amphotericin B or fluconazole it is important to assess the severity of the infection at the time of diagnosis. Factors in earlier studies indicating an unfavorable outcome, were not able to predict poor outcome in our, albeit small, group of patients.[6,10,11] The only factor we found to be clearly predictive of a complicated course of cryptococcal meningitis was a high CSF opening pressure at the time of diagnosis, and 30 cm H_2O seemed to be a critical limit in this study. In earlier studies, in the majority of patients, the CSF opening pressure was not determined.[6-8] Denning et al. suggested inadequate treatment of high intracranial pressures to be responsible for the loss of vision and early mortality in cryptococcal meningitis.[12] However, they did not evaluate the prognostic value of the CSF opening pressures.

The variety of treatment regimes may be partly responsible for the differences in clinical outcome in this study; all three patients treated with fluconazole had a complicated course of infection. One patient died on the first day; the other two experienced seizures on the 3rd and 5th day of fluconazole treatment. Especially in the case of the first two patients it is unlikely that the course of the infection would have been substantially different if another antifungal therapy had been used.

The results of this study suggest that a CSF opening pressure above 30 cm H_2O is an important prognostic factor of the outcome of cryptococcal meningitis in HIV-infected patients. The real value of raised intracranial pressure should be evaluated in a prospective study as is currently conducted in the USA. For the time being we recommend to measure the CSF opening pressure when performing a lumbar puncture in these patients. If the CSF opening pressure is >30 cm H_2O at the time of diagnosis, one should consider serious disease that should preferably not initially be treated with azoles. If high pressures persists, specific measures such as repeated lumbar punctures or the insertion of an external lumbar drain should be considered in order to lower this pressure.[12]

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Chapter 9

AmBisome compared with amphotericin B both followed by oral fluconazole in the treatment of AIDS-associated cryptococcal meningitis.

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ABSTRACT

Amphotericin B deoxycholate (Amb) initial therapy and fluconazole maintenance therapy is the treatment of choice for AIDS associated cryptococcal meningitis. However, the administration of Amb is associated with considerable toxicity. A potential strategy for reducing the toxicity and increasing the therapeutic index of Amb is the use of lipid formulations of this drug.

Design and methods. HIV-infected patients with cryptococcal meningitis were randomized to treatment with either liposomal amphotericin B (AmBisome®) 4 mg/kg/day or Amb 0.7 mg/kg/day for three weeks, each followed by fluconazole 400 mg/day for seven weeks. During the first three weeks clinically efficacy was assessed daily. Mycological response was primarily evaluated by cerebrospinal fluid (CSF) cultures at day 7, 14, 21 and 70.

Results. Of the 28 evaluable patients, 15 were assigned to receive AmBisome and 13 to receive Amb. Baseline characteristics were comparable. The time to and the rate of clinical response were the same in both arms. AmBisome therapy resulted in a CSF culture conversion within seven days in 6/15 patients versus Amb in 1/12 (P=0.09), within 14 days in 10/15 patients versus 1/9 (P=0.01), and within 21 days in 11/15 patients versus 3/8 (P=0.19). When Kaplan-Meier estimates were used to compare time to CSF culture conversion, AmBisome was more effective (P<0.05) (median time between 7 and 14 days for AmBisome versus >21 days for Amb). AmBisome was significantly less nephrotoxic.

Conclusion. A three week course of 4 mg/kg AmBisome results in a significantly earlier CSF culture conversion than 0.7 mg/kg Amb, has equal clinical efficacy and is significantly less nephrotoxic when used for the treatment of primary episodes of AIDS associated cryptococcal meningitis.

INTRODUCTION

Although the incidence is decreasing, cryptococcal meningitis is still the most common manifestation of systemic fungal infection in HIV infected patients and remains associated with significant morbidity and mortality.[1,2,3] Amphotericin B deoxycholate (Amb) given for a period of 4-6 weeks has been considered to be the gold standard for the initial treatment of cryptococcal meningitis in patients with HIV infection. Initial treatment with the triazoles fluconazole and itraconazole is probably less effective.[4,6] Unfortunately, the administration of Amb may be associated with significant toxicity, mostly nephrotoxicity and acute infusion related reactions. In order to reduce the risk of Amb-related toxicity, many clinicians have attempted to use shorter courses of Amb followed by oral triazoles in the treatment of AIDS-associated cryptococcal meningitis. A study examining initial treatment with 0.7 mg/kg/day Amb with or without flucytosine for two weeks, followed by fluconazole or itraconazole has recently been completed in the U.S.[7,8]

Another potential strategy for reducing Amb-associated toxicity is the use of liposomal and other lipid-based formulations containing amphotericin B.[9-12] These formulations have shown a reduced toxicity and have permitted the use of higher doses of amphotericin B, which potentially could lead to an increased therapeutic index.[13] One of such liposomal formulations is AmBisome (NeXstar Pharmaceuticals Inc., San Dimas, California), which has demonstrated promising results in a phase II study for the treatment of AIDS-associated cryptococcosis.[14] We performed an open randomized trial in HIV infected patients with a primary episode of cryptococcal meningitis comparing a three-week course of Amb 0.7 mg/kg/day with AmBisome 4.0 mg/kg/day, followed in both arms by treatment with fluconazole 400 mg/day for an additional seven weeks.

METHODS

Study Population. Hospitalized HIV infected patients, 18 years of age or older, with a primary episode of cryptococcal meningitis were eligible for enrollment. Written informed consent had to be given by the patient or his legal guardian. Patients could be enrolled either on the basis of a positive India ink stain or the presence of cryptococcal antigen (CRAG) in the cerebrospinal fluid (CSF), while culture results were pending. Following enrollment, confirmation of cryptococcal

meningitis was required either by a positive CSF culture for *Cryptococcus neoformans*, or a positive test for CRAG in CSF together with a positive blood culture for *C. neoformans*. Patients were excluded if they had already been treated for cryptococcal infection or if serum creatinine was above 250 μmol/L.

The study protocol was reviewed and approved by the ethics committees of all participating Dutch and Australian centers, and by the National Aids Therapy Evaluation Centre in the Netherlands and the National Centre in HIV Epidemiology and Clinical Research in Australia.

Treatment. Patients were randomized 1:1 to receive a 3-week monotherapeutic regimen of either AmBisome (4 mg/kg/day i.v.) or Amb (0.7 mg/kg/day i.v.). Randomization was performed by means of sealed envelopes, centrally by one Dutch and one Australian coordinating centre and was stratified by participating site. All patients completing these initial three weeks of study treatment while hospitalized, continued treatment with fluconazole (400 mg/day orally) for seven weeks. Subsequently fluconazole 200 mg was recommended to prevent relapses of cryptococcal infection; all patients were followed-up at least for six months to document relapses.

AmBisome was administered at full, without using a dose escalation schedule, and was infused over 45 minutes; no central intravenous catheter was required. For amphotericin B the dose was escalated within 24 h to a full dose of 0.7 mg/kg/day, infused over six h. The use of a central intravenous catheter was recommended. Sodium supplementation prior to Amb infusion to prevent nephrotoxicity was recommended and administration of concomitant medication to prevent acute reactions was allowed.

Dosage adjustments were required when serum creatinine rose to $\geq$ 300 % of a normal or $\geq$ 200 % of an already elevated baseline value. Study medication was then to be discontinued for 2 days and subsequently could be reinstituted at half dose, when creatinine levels had fallen below 300% or 200% of the base line values, respectively. If a rise in serum creatinine did not recur, doses were escalated to full. Study medication had to be discontinued if serum creatinine levels had not fallen below the above mentioned levels after five days administration of reduced doses. Dose adjustment was not mandated in case of elevation of liver enzymes.

Clinical Evaluation. Patients were evaluated daily during the first three weeks of study treatment for vital signs, temperature, degree of headache (absent, mild, moderate, intractable), Glasgow Coma Scale score and the pres-

ence of meningeal symptoms. During this period an additional neurological and general physical examination as well as determination of the Karnofsky score were performed weekly. During the next seven weeks of treatment with fluconazole, patients were similarly examined at least every two weeks as out-patients. The following criteria were used for clinical evaluation: 1) Clinical response: normalization of all above mentioned pre-treatment signs, symptoms and scores. 2) Time to clinical response. 3) Clinical relapse: following clinical response, recurrence of any signs or symptoms of cryptococcal meningitis during the 10 week study period or the six month follow-up.

Blood urea nitrogen, serum creatinine and potassium were determined pre-treatment and twice weekly and additional chemistry (including liver enzymes) and hematologic determinations weekly during the first three weeks and every two weeks until week 10.

Study drug treatment was prematurely discontinued in case of a sustained rise in serum creatinine levels (see above), in case of other serious adverse events judged to be study drug related by the treating physician or if requested by the patient or physician.

The following factors were studied for being predictive for clinical outcome: altered metal state, positive extra neural cultures, positive blood cultures, lumbar opening pressure > 30 cm H_2O, CSF white blood cell (WBC) count, CSF cryptococcal antigen (CRAG) titer and serum CRAG titer. All factors were included in the multivariate analysis.

Mycological evaluation. CSF (5ml), blood (10-20 ml) and material from all other sites suspect for cryptococcal infection (e.g. urine, sputum, lymph node) were obtained before starting study treatment. Lumbar punctures were repeated after 7, 14 and 21 days and after 10 weeks. An additional puncture was performed at day 28 in those patients whose CSF culture at day 21 was the first to remain sterile. Any other previously positive cultures were repeated. In addition, CSF WBC counts, protein, glucose and CRAG titers were determined and lumbar opening pressure was recorded. Serum CRAG levels were measured weekly. Determination of CRAG titers was performed locally at each participating center using several tests: Pastorex (Sanofi Diagnostics Pasteur), CALAS (Meridian Diagnostics) and Immy (Immunomycologics). All these tests were performed at the local laboratories. The following criteria were used for mycological evaluation: 1) Mycological response: CSF culture conversion, meaning the achievement of two consecutive negative cultures from the CSF. 2) Time to mycological

response: time to the achievement of the first of two consecutive negative cultures from the CSF. 3) Persistence: after 10 weeks of study medication at least one culture from a specific site had remained positive. 4) Mycological relapse: Following a CSF culture conversion, recurrence of a positive culture during the remainder of the study or the six-month follow-up period.

Overall evaluation. Overall response: both clinical and mycological response at the completion of the 10-week treatment period.

Serum and CSF concentrations of amphotericin B. Trough and peak serum samples and CSF samples were obtained after 7, 14 and 21 days of therapy respectively. The samples of CSF were obtained randomly just before or after infusion of the study drug. Levels of amphotericin B were batchwice measured by High Performance Liquid Chromatography at the University Hospital Rotterdam, as previously described, which assay has a sensitivity of 0.1 μg amphotericin B per ml blood.[15]

Study design and statistical analysis. The study was primarily powered to show the superiority of AmBisome over Amb in achieving CSF culture conversion more rapidly. Assuming a CSF culture-conversion rate of 90% for AmBisome versus 40% for Amb after one week of therapy, a sample size of 13 evaluable patients in each arm was expected to be sufficient to enable significant conclusions (two-sided p=0.05, power=80%).[5,14] Time to clinical and mycological response was calculated according to the method of Kaplan and Meier. Comparison of these curves was done with the log-rank test. Percentages were compared by Fisher's exact test. Continuous variables were compared by Student's t-test. Cox regression was used for multivariate analysis of factors prognostic for clinical outcome. Correlation coefficients given are Spearman's. The change from baseline of logarithmically transformed creatinine values was compared using repeated measurements analysis of variance (RmANOVA).[16] A p-value $\leq$ 0.05 was considered significant.

All analysis of clinical and mycological efficacy data were performed on an intention-to-treat basis. For analysis of toxicity, patients were evaluated up to the time of definite discontinuation of study drug.

RESULTS

Study population. Between June 1992 and June 1995, 26 HIV infected patients with suspected cryptococcal meningitis (25 on the basis of a positive India ink

stain and one with a positive CRAG test of CSF), and four HIV infected patients with culture proven cryptococcal meningitis were enrolled in the study. Two patients were subsequently found to be ineligible. One comatose patient was randomized to AmBisome in error as no informed consent from a legal guardian had been obtained. The other patient, assigned to Amb, was withdrawn because of subsequent negative cultures. Twenty-eight patients were further analyzed. Fifteen were assigned to AmBisome and 13 to Amb. At entry, clinical parameters and laboratory test results were comparable (Table 1), although patients in the AmBisome group had a significantly higher serum CRAG titer (P=0.007 by Student's t-test); CSF CRAG titers were comparable in both groups. No differences were found when results from the different centers or tests were compared. There were no differences in pretreatment clinical characteristics or laboratory test results between the patients in Australia and the Netherlands. All 28 evaluable patients are included in the intention-to-treat analysis for efficacy.

Premature discontinuation of treatment. Five patients did not receive the full three-week course of study treatment. Two AmBisome treated patients were switched to other medication early (both at day 14), one because of persistently elevated CSF pressure and the other because of mild somnolence possibly related to disease progression or toxicity of the study medication. One patient treated with Amb was withdrawn prematurely (day 14) because of chorioretinitis, judged to be a sign of progression of cryptococcal infection. Two other patients treated with Amb were withdrawn because of laboratory toxicity (see toxicity section). Two of the 23 patients who did complete three weeks of study medication (both randomized to AmBisome), were not switched to fluconazole because of persistently positive CSF cultures with *C. neoformans*, but first received the combination Amb with flucytosine for three and four weeks respectively. One patient assigned to Amb was lost to follow up eight weeks after enrollment.

Clinical outcome. Clinical response rates after the first three weeks of treatment were 12/15 (80 %; 95 percent confidence interval, 52 % - 96 %) and 11/13 (86 %; 95 percent confidence interval, 55 % - 98 %) in the AmBisome and Amb treated groups respectively (P=1.0). The median time to clinical response was 15 days in both arms. No patients died during this period. During the subsequent seven treatment weeks one patient treated with AmBisome (at week 6), and two treated with Amb (at week 7 and 10 respectively) died. Clinical response rates at week 10 were 13/15 patients (87%) assigned to AmBisome and 10/12

(83%) patients assigned to Amb. The one remaining patient assigned to AmBisome who had failed to respond clinically to therapy, achieved a clinical response at week 14. There were no clinical relapses observed in the 10 weeks study period. No proven clinical relapses occurred during the six-month or further follow up. One patient who had been treated with AmBisome died, after having been

Table 1. *Baseline clinical characteristics and results of laboratory tests in 28 HIV-infected patients with cryptococcal meningitis.*

Characteristic	Amphotericin B deoxycholate (n=13)	AmBisome (n=15)
Median age in years[a]	41 (28-49)	40 (29-55)
Antiretroviral drug use	11	7
Previous AIDS diagnosis	6	7
Signs and symptoms		
headache	11	15
nausea/vomiting	6	9
fever (>38°C)	6	6
meningismus	3	6
altered mental state	2	6
Glasgow Coma Scale score[a]	15 (14-15)	15 (12-15)
Karnofsky score	70 (30-80)	70 (30-90)
Laboratory tests		
Blood		
CD4 cell count (cells/mm^3)[a]	35 (20-90)	35 (10-70)
CRAG titer*	512 (40-2,048)	5,120 (512-256,000)[b]
CSF		
Opening pressure (cm/H_2O)[a]	23 (13-65)	26 (10-39)
White cell count (cells/mm^3)[a]	7 (0-67)	5 (1-94)
Protein (g/L)[a]	51 (25-104)	79 (44-844)
Glucose (mmol/L)[a]	2.5 (1.4-3.2)	2.8 (0.1-3.9)
CRAG titer[a]	256 (8-32,768)	1,024 (2-256,000)
Extraneural culture positive		
Blood	7/11	7/12
Urine	1/5	6/9
Other[c]	0	3
Ptt with poor prognostic marker[d]	8/13	11/15

[a]Data are expressed as medians followed by ranges (in parentheses).
[b]*P*=0.007 by Student's t-test when compared to amphotericin B deoxycholate treated patients.
[c]Broncho-alveolar lavage fluid, sputum, lymphnode.
[d]Opening pressure > 30 cm H_2O, CSF WBC < 20 cells/mm^3 or CSF CRAG titer > 1024.

admitted to hospital with a reduced level of consciousness, for which no definite explanation was found. During the six-month follow-up two additional patients died (one in each arm); both not as a result of cryptococcal disease.

Factors predicting outcome. Both in univariate and multivariate analysis two factors were shown to be significantly associated with a longer time to clinical response; a CSF WBC count of < 20 cells/mm^3 and a raised lumbar opening pressure > 30 cm H_2O (Table 2).

Mycological outcome. AmBisome therapy resulted in a CSF culture conversion within seven days in 6/15 patients versus Amb in 1/12 (*P*=0.09). Significantly more patients treated with AmBisome had a CSF culture conversion within 14 days (10/15 patients) when compared with patients treated with Amb (1/9 patients) (*P*=0.01). Within 21 days 11/15 patients treated with AmBisome versus

Table 2. *Factors significantly associated with a longer time to clinical response in AIDS associated cryptococcal meningitis.*

Characteristic	univariate analysis		multivariate analysis	
	median time to clinical response (days)		relative response rate (95 percent CI)	
CSF WBC ≤20 cells/mm^3	16		1	
CSF WBC >20 cells/mm^3	11	(*P* = 0.04)	4.5 (1.4 - 17.9)	(*P* = 0.008)
LOP > 30 cm H_2O	21		1	
LOP ≤ 30 cm H_2O	14	(*P* = 0.03)	4.9 (1.5 - 13.3)	(*P* = 0.018)

CSF=cerebrospinal fluid, WBC=white blood cell count, LOP=Lumbar Opening Pressure

3/8 treated with Amb had responded mycologically (*P*=0.18). Five patients assigned to Amb did not underwent all scheduled lumbar punctures, because of abnormalities in hemostasis (1), refusal (2) and unintentional omission (2). These patients were not scored as being failures, but were censored in the analysis, explaining the different denominators in week 1, 2 and 3. When Kaplan-Meier estimates were used to compare time to CSF culture conversion, AmBisome was

significantly more effective than Amb ($P<0.05$) (Fig. 1). The median time to CSF culture conversion was between seven and 14 days for AmBisome versus >21 days for Amb. One patient treated with AmBisome with negative CSF-cultures at week one and two, had a mycological relapse at week three, but was nevertheless successfully switched to fluconazole according to the study protocol. In the patients who underwent lumbar punctures after 10 weeks of treatment, 11/11 patients initially treated with AmBisome and 8/8 patients with Amb had negative cultures. No mycological relapses were noted during the six month and further follow-up period.

Blood cultures for cryptococcus became negative after a median of seven days in both treatment groups. All other cultures taken from extra-neural sites which had been positive at enrollment (Table 1), became negative within 21 days.

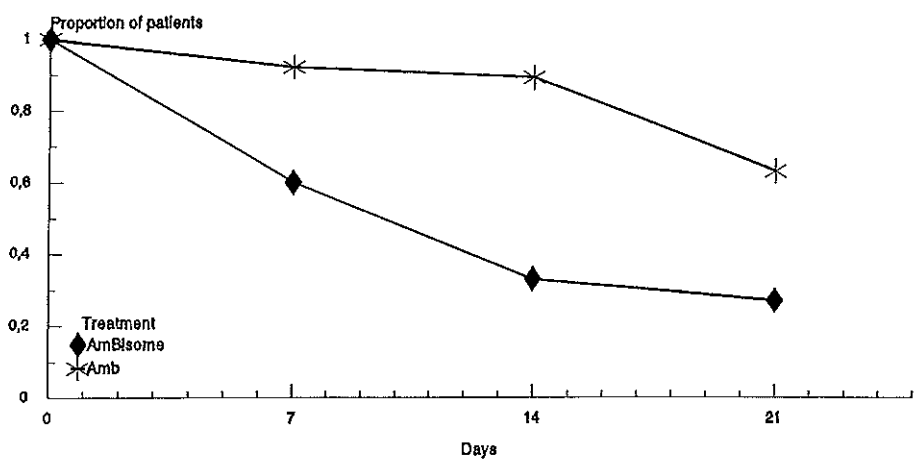

Figure 1. *Kaplan-Meier estimates of the proportion of patients with positive cerebrospinal fluid cultures during the first 3 weeks of treatment, according to treatment group.*

CRAG titers in CSF showed a consistent decrease throughout the first three weeks of treatment with no differences between the two treatment groups. Serum CRAG titers in both groups remained at about the same level throughout this period. Lumbar openings pressures showed a consistent decrease in both arms throughout the first three weeks of treatment. No relation between clinical or mycological response and the course of lumbar opening pressures was found.

A significant correlation was found between the time to CSF culture conversion and the time to clinical response (r=0.63; P<0.001) (Fig. 2).

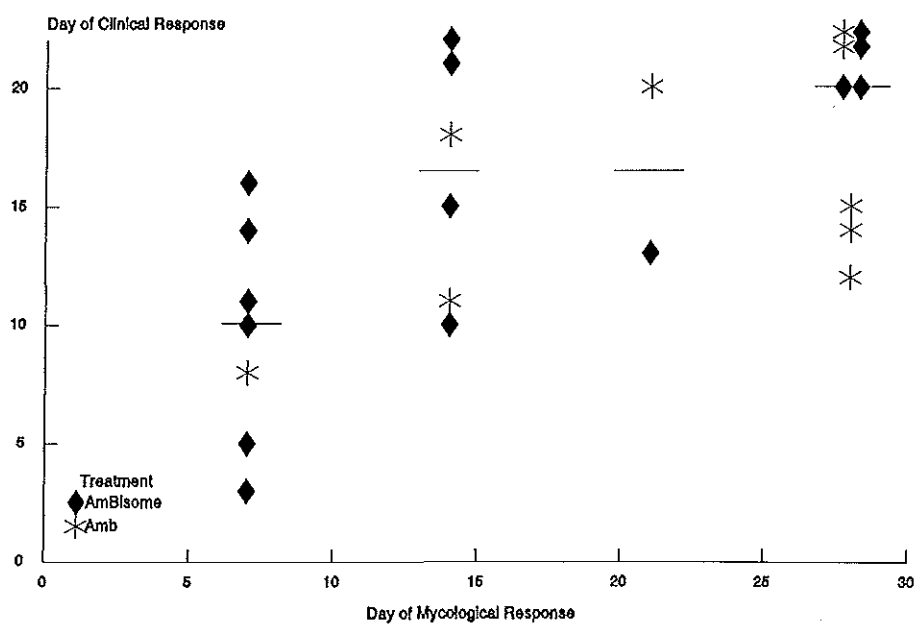

Figure 2. *Correlation between the time to mycological response and the time to clinical response. The times to mycological and clinical response show a significant correlation in AIDS-patients with cryptococcal meningitis treated with AmBisome of amphotericin B (P=0.0009 by Spearman rank correlation test).*

Table 3. Number of patients with adverse events during treatment.

Adverse Event	Amphotericin B deoxycholate (n=13)	AmBisome (n=15)
Clinical		
Chills	2	0
Nausea	1	0
Thrombophlebitis	1	0
Somnolence	0	1
Other acute reactions[a]	0	2
Detected in laboratory		
Fall in Hemoglobin > 2.0 g/dL	6	4
Hypokalemia (< 3.0 meq/L)	4	3
Creatinine > 3 times ULN[b]	1	0
Aminotransferase > 5 times ULN	3	1
Alkaline Phosphatase > 5 times ULN	2	0
Bilirubin > 5 times ULN	3	1
None of these adverse events reported	3[c]	8[c]

[a]See results.
[b]ULN = Upper Limit of Normal.
[c]*P*=0.14 (Fisher's exact test).

Toxicity. Both treatment regimens were generally well tolerated (Table 3). Three patients experienced clinical adverse events probably or possibly related to AmBisome. Mild somnolence was observed in one patient after two weeks of therapy. Two patients developed acute reactions during the first infusion of AmBisome. Tachycardia, hypotension, facial flushing and a tickling cough was observed in one patient, and tachycardia, shortness of breath and fever in the other. In both patients these reactions disappeared when infusion was interrupted and did not recur when the infusion continued at a lower infusion rate. Concerning nephrotoxicity; when increases from baseline of serum creatinine levels at the various time points were analyzed with RmANOVA, it was found that this increase was on the average a factor 1.37 (*P*=0.003) greater in the Amb treated patients. Three patients treated with AmBisome and four patients treated with Amb experienced hypokalemia, but none of these patients had to discontinue therapy for this reason. The number of patients with major changes in bio-

chemical parameters are shown in Table 3; the median percentages increase in Table 4. Only the median percentage increase in bilirubin was significantly smaller in patients treated with AmBisome (Table 4). Two patients treated with Amb were withdrawn because of laboratory toxicity; withdrawal of one patient (at day 10) was protocol mandated by an increase in serum creatinine, and of the other (at day 14) by an increase in liver enzymes.

Table 4. *Changes in hemoglobin, Aspartate transaminase (AST), Alanine transaminase (ALT), alkaline phosphatase and bilirubin after 3 weeks of therapy (median and range of percentages decrease or increase from baseline).*

parameter	Amphotericin B deoxycholate	AmBisome	*P*-value
hemoglobin	-20.0 (-45.2-4.6)	-20.8 (-49.5--4.5)	0.97
AST	11.0 (56.1-130)	-13.5 (-79.5-125)	0.32
ALT	17.8 (-60.2-411)	53.5 (-75.2-350)	0.90
alkaline phosphatase	27.1 (-21.9-124)	10.9 (-52.0-86.5)	0.20
bilirubin	10.0 (-71.4-221)	-25.0 (-58.3-41.7)	0.05

Serum and CSF concentrations of amphotericin B. Median serum trough levels (17.6 μg/ml, range 2.4-65.6) and peak levels (44.4 μg/ml, range 20.6-100) of amphotericin B in patients treated with AmBisome, were higher than those in
patients treated with Amb (0.9 μg/ml, range 0.5-2.1) and (1.8 μg/ml, range 1.2-5.2) respectively (both $P<0.001$). However, in none of the samples of CSF obtained just before or after infusion with either AmBisome or Amb, amphotericin B levels could be detected.

DISCUSSION

Our study is the first randomized comparative trial to show superior antifungal activity of liposomal amphotericin B (AmBisome) over standard Amb in the treatment of a specific fungal infection. Liposomal or lipid based formulations of

amphotericin B had already been shown to be less toxic than Amb and might have enhanced antifungal efficacy when high dosages are used. Indeed, an improved therapeutic index has been reported in several animal models, including models for cryptococcosis.[13,17-20] In patients with AIDS-associated cryptococcal meningitis, a randomized study comparing up to 5 mg/kg Abelcet with 0.7-1.2 mg/kg Amb showed no evidence of improved activity.[21] Based on differences in pharmacologic properties however, the results obtained for one of the lipid-based formulations of Amb cannot be applied to other formulations, unless comparative studies are available.[10,11]

In our study, two thirds of patients given AmBisome, had a CSF culture conversion within 14 days, more than half of these patients even within seven days of treatment. In comparison, the median time to CSF culture conversion was more than 21 days for patients assigned to Amb, with only a single patient having achieved CSF culture conversion within seven days. Unfortunately, several patients in the Amb group did no undergo a lumbar puncture at day 14 and 21. However, we feel that this has probably not influenced the overall outcome of our study, for time to clinical response of these patients was the same as in patients who underwent all lumbar punctures (median time 14 days (range 10-19 days). Furthermore these patients were not scored as failures, what should have been appropriate in an intention to treat analysis, but were censored, so they could not negatively influence the results of the Amb group. Our findings are remarkable when considering that the median time to the first negative CSF culture was as long as 42 days for patients treated with Amb in the largest trial of AIDS-associated cryptococcal meningitis published to date, although only moderate doses were used.[4] The short median CSF culture conversion time following AmBisome treatment could not be explained by a predominance of less severe cases. The severity of illness in our patient population as indicated by factors known to be predictive of worse outcome (altered mental state, a lumbar opening pressure > 30 cm H_2O, a CSF WBC count < 20/mm^3 and a CSF CRAG titer > 1:1024) was comparable to that in other studies.[4,5,22] The relatively low number of Amb treated patients Amb who had a CSF culture conversion at day 14 when compared to the most recent ACTG trial might be explained by the fact that we included patients with severe infections as well.[7] We could not explain the differences which were found in the serum CRAG titers; they were not correlated to positive blood cultures, nor to the CRAG titer in the CSF. However, high CRAG titers are only known to be predictive for a worse

outcome in non HIV-infected patients but not in AIDS patients with cryptococcal meningitis, so we do not consider this to be a confounding factor.[23]

Our strategy of initiating therapy with intravenous Amb, and consolidating with an oral triazole antifungal agent is very similar to the one used in a large trial of AIDS-associated cryptococcal meningitis, which was recently completed.[7,8] The high rates of clinical and mycological responses in our study after ten weeks of treatment, as well as the low mortality during the initial period of intravenous therapy, is very comparable to the overall results obtained for patients in this study who had received fluconazole as consolidation therapy. We are not aware of reports concerning the influence of the addition of flucytosine to AmBisome, but this might be an option to further increase efficacy. We have not been able to show a superior clinical response for patients receiving AmBisome, but this may well be due to the limited number of patients in our study.

As has been shown for Abelcet, AmBisome was significantly less nephrotoxic than Amb in this group of patients.[21] Although, as in patients treated with Amb, hypokalemia was frequently observed in the patients receiving AmBisome, it did not lead to premature discontinuation of treatment in any of these patients. This study also showed that high doses of AmBisome can be given to HIV patients without severe adverse events. More than half of the patients treated with AmBisome did not experience any side effect during the three-week treatment. Coker et al. showed that during treatment with AmBisome changes in liver enzymes occurred.[14] We showed that there was no significant difference in changes in liver enzymes between patients treated with AmBisome and Amb. The minor but significant difference between changes in bilirubin, may have been caused by the interference of lipids in the determination of the levels of bilirubin.[24] The only other relevant toxicities were observed in two patients during the first infusion of AmBisome. However, restarting drug administration at a lower infusion rate was uncomplicated. These events are quite similar to the adverse events described by Arning et al.[25] We therefore suggest that patients who receive AmBisome for the first time, should be closely monitored during this infusion. When acute infusion-related reactions occur, infusion should be stopped but can be restarted at a lower rate.

Although serum peak and trough levels of amphotericin B in patients receiving AmBisome were much higher than in those receiving Amb, the relevance of this finding is unclear as the assay used can not distinguish between lipid-bound and free amphotericin B.[10] Since no levels of amphotericin B in CSF

were detectable, apparently this agent is bound to the meninges. This is consistent with the observation in animals treated with AmBisome.[26] The lack of correlation between the presence of amphotericin B in the CSF and the outcome of cryptococcal meningitis following treatment with Amb and other amphotericin B containing formulations, is understandable if one considers this to be an infection of the brain and meninges. Large numbers of cryptococci can indeed be seen in tissue sections of brain and meninges from patients with cryptococcal meningitis.[27] A similar lack of correlation has been reported for itraconazole.[28]

An often mentioned significant drawback of AmBisome and other lipid-based formulations of amphotericin B is their high cost.[11,29] However, as we have demonstrated a significant correlation between the time to CSF culture conversion and the time to clinical response, with CSF culture conversion being accomplished in a substantial proportion of AmBisome treated patients within as little as seven days, short treatment with AmBisome (e.g. for one week), followed by oral fluconazole may be appropriate. Reduction of the duration of hospitalization, low toxicity rates, the shortened duration of infusion and the possibility of administering AmBisome through peripheral veins could result in a cost-effective regimen. We suggest that comparative studies should address these possibilities and should include a formal cost benefit analysis for the use of AmBisome in the treatment of AIDS associated cryptococcal meningitis.

Our results indicate that a high dose AmBisome regimen combines a reduced toxicity with an increase in antifungal activity compared to standard Amb in HIV infected patients with cryptococcal meningitis, when used in a dose as high as 4 mg/kg/day for three weeks.

The AmBisome regimen used in this study is probably the most effective single agent regimen for the treatment of AIDS associated cryptococcal meningitis.

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Chapter 10

General discussion

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GENERAL DISCUSSION

The incidence of opportunistic fungal infections has been increasing and may well increase further in the near future.[1,4] This can be expected if the number of susceptible patients increases, for example with patients that receive transplants and HIV-infected patients. However, other developments in medicine may bring some relieve from this trend. The new anti-retroviral treatment regimens have been shown to decrease the incidence of opportunistic infections in HIV-infected patients. Alterations in immunosuppressive therapy may soon follow. However, safe and highly effective antifungal agents are still not available today. The ongoing search for new, more active and less toxic drugs has resulted in the development of new agents and new (lipid-based) formulations of older agents.[5-10] The exact place of these new agents and formulations has not yet been established, but none of them seems to be the magic bullet against fungi. Therefore, prevention of invasive fungal infections in immunocompromised patients remains an extremely important strategy and studies are conducted to learn about the epidemiology of these infections to develop appropriate preventive strategies.

The **aim of the studies** presented in this thesis was to gain further insight in the epidemiology of opportunistic fungal infections and to compare the toxicity and efficacy of one of the lipid-based formulations of amphotericin B (AmB) to that of AmB in the treatment of these infections.

Epidemiology

Aspergillus spp. are ubiquitously present in dust, soil, water etc. Most cases of invasive aspergillosis among granulocytopenic patients are pulmonary infections. This reflects the fact that the lungs are the major portal of entry for aspergilli. Several sources for aspergilli have been described, inside as well as outside the hospital.[11-15] To gain further insight in the epidemiology of nosocomial invasive aspergillosis, an apparent outbreak of invasive aspergillosis at our hematology department was investigated in depth (chapter 2), and a surveillance program for fungal conidia inside the hospital and in the outside air was conducted thereafter (chapter 3). The initial question while investigating such an outbreak is whether it is due to a source of fungi in the building in question or to an increased introduction of fungi into this area. We studied if genotyping of the available fungal isolates could be used to answer this question. Although no cause could be determined for the apparent outbreak of invasive aspergillosis, we showed that fingerprinting by means of randomly amplified polymorphic DNA (RAPD) was

helpful to focus further investigations. We also showed the importance of using the right type and number of RAPD-primers to obtain satisfactory discriminative power, when using this technique (chapter 2). From the fingerprinting patterns of clinical and environmental isolates, it became clear that the outbreak was probably not caused by a single source such as for instance fungal growth in rotting wooden materials in the patient wards, but rather by the introduction of conidia either from sources elsewhere within or outside the hospital. Further investigations could therefore be focussed on leakages in barrier systems and proper functioning of the air-conditioning systems. Genotyping of environmental isolates obtained during surveillance of the air in the department of hematology showed that some clones of *A. fumigatus* were able to remain present at this department for periods of more than a year (chapter 3). Because almost all environmental isolates from this department belonged to one of these clones, and because they were all genotypically different from the patients isolates, these patients were probably infected with strains not derived from the hospital air. This raises the possibility that some patients develop invasive aspergillosis with strains already colonizing their airways at the time they enter the hospital. If this is correct, barrier procedures after admission will never suffice to prevent all cases of invasive aspergillosis. Prophylactic antifungal therapy with activity against *Aspergillus* spp. may have to be offered to patients that receive myelo-suppressive chemotherapy. Furthermore, one should consider the possibility to admit patients to HEPA-filtered rooms some time before they actually receive chemotherapeutic courses, in order to "wash out" conidia already present in their airways and preventing new strains to do so just prior to immunosuppressive treatment. It may even be wise to take preventive measures when patients are dismissed between courses or to take surveillance cultures at home.

Another way of "preventing" the clinical consequences of full blown invasive fungal infections is the institution of pre-emptive antifungal therapy.[16] Sensitive diagnostic tests are needed that can detect the early stages of the disease process such that a secondary preventive strategy becomes feasible. This may be possible in the future if diagnostic assays including ELISA's and nucleic acid techniques prove to be as sensitive as claimed in early reports.[17-19] Clinical trials should then determine which antifungal agents are most suitable for this purpose.

Despite the aforementioned issues, studies have shown that barrier procedures, especially nursing patients in HEPA-filtered rooms, are helpful and will diminish the incidence of invasive aspergillus infections.[20,21] Results from our

surveillance study show that the measurement of conidia of non-pathogenic fungi, which are present in 10-fold higher densities, can be taken as sensitive parameter in the control of barrier systems (chapter 3). An increase in densities of conidia should induce an extensive search for the cause of this increase. Genotyping by means of RAPD can help to distinguish whether one should search for a common source of fungal growth (when genotypically identical isolates are found) or for a leak in the barriers (genotypically different isolates).

A molecular typing study of cryptococci showed that RAPD can be used for the typing of other fungal pathogens as well and that the results match the results of other (geno-)typing methods (chapter 4). The RAPD patterns of *C. neoformans* from patients treated in Rotterdam showed less variation when compared to the patterns obtained with *Aspergillus* spp. (chapter 2). Because pulsed-field gel electrophoresis (PFGE) resulted in the same variation it was suggested that clonal expansion of this *C. neoformans* has occurred in this restricted region. The proportion of HIV-infected patients that become infected with *C. neoformans* varies largely between different regions of the world. No explanation is available for this difference in global prevalences. If clonally related isolates are present in other restricted geographic regions as well, differences in virulence between these isolates could explain part of the difference in prevalence of cryptococcosis in HIV-infected patients.[22] However, these differences can also be due to an increased contact of HIV-infected persons with the pathogen in that particular area of the world.

Management

Even today the number of drugs that are effective in the treatment of invasive fungal infections remains very limited. The most widely used drug (AmB) has major side effects, and failure rates remain high. This underscores the importance of developing new antifungal drugs and formulations. One of the promising results in the search for new drugs was the development of triazoles, of which fluconazole and itraconazole are already frequently used.[5,6,23,24] The major advantage of these products is their relative lack of toxicity. However, azoles also have some disadvantages. First, these drugs are said to be fungistatic, and, therefore, not recommended for primary use in immunocompromised patients.[25] An example is the use of fluconazole or itraconazole as primary therapy for cryptococcal meningitis in HIV-infected patients, which resulted in a longer cerebrospinal fluid (CSF)-culture conversion time than when AmB was used.[26-28] In addition more relapses were noted, which also indicates that cryptococci are

not sufficiently eradicated by azole exposure. In contrast, in a comparative study of fluconazole and AmB in patients with invasive candidiasis, fluconazole showed more favorable results only in a subgroup of neutropenic patients, so the debate on the use of azoles in immunocompromised patients is ongoing.[29]

Itraconazole is only available as an oral formulation, which unfortunately yielded unpredictable serum levels particularly in neutropenic patients.[24] A recently introduced oral liquid formulation promises improvement in bio-availability. The spectrum of activity of both triazoles differs widely. Itraconazole has a broad spectrum of activity in contrast to fluconazole. Fluconazole is mainly active against yeasts, and other agents should be studied as empiric therapy for neutropenic patients with fever of unknown origin in a setting where *Aspergillus* infections are a problem or when chest X-rays show abnormalities.[16]

A final problem is the "emergence" of azole-resistent fungal pathogens. First there is an increase of *Candida* non-albicans such as *C. krusei* and *C. glabrata* which are intrinsically resistent to azoles.[30,31] Furthermore, *C. albicans* isolates with a decreased susceptibility for azoles have been isolated after prolonged therapy with azoles and even itraconazole resistent *Aspergillus* spp. have been described.[32,33] Because of this trend, there is a clear need for standardized susceptibility tests both for yeasts and molds. The NCCLS has developed such a standard for susceptibility testing of yeasts, however, this method is not an "easy to use" one.[34] Methods that are easier to perform (such as E-test) and that may correspond to this standard have also been described.[34] At this moment unfortunately, no standard is available for the susceptibility testing of moulds.

Because of the aforementioned, AmB still remains the therapy of choice for many fungal infections especially in immunocompromised patients. To overcome problems with its toxicity, lipid-based formulations of AmB have been developed (chapter 5). Three such formulations have been marketed: Amphotericin B Lipid Complex (ABLC, Abelcet), Amphotericin B Colloidal Dispersion (ABCD, Amphocil, Amphotec) and liposomal amphotericin B (AmBisome). These three formulations differ significantly in their composition and pharmacokinetics.[35] All three formulations share a considerable reduction in the incidence of AmB-related nephrotoxicity, but the number of acute reactions differ among these compounds. In the early nineties, no clinical trials comparing lipid-formulations of AmB with AmB had been published or presented. Therefore, these compounds were only recommended as salvage treatment for cases of intolerance to or failure on AmB therapy.

The efficacy of all three formulations was first studied in animal models for several fungal infections.[36-47] To compare the efficacy of AmBisome to that of AmB we developed a rat-model of invasive pulmonary aspergillosis that mimicked human disease during neutropenia; one-sided onset of infection of the lung, persistent granulocytopenia and start of treatment at a time when hyphal growth is firmly established (chapter 6). In this model, no differences in efficacy in terms of delayed or reduced mortality were found between high doses of AmBisome and AmB. Lower doses of AmBisome (equal to the optimal dose of AmB) were not able to reduce mortality as compared to untreated animals. Therefore, the use of these doses in a therapeutic was questioned. Most animal-models comparing lipid formulations of AmB with AmB show similar results; doses equal to the optimal dose of AmB being less effective than AmB while the highest doses of lipid formulations often show better efficacy (chapter 5). However, in contrast to AmB, in our model both high and low doses of AmBisome significantly diminished dissemination of infection. Because AmBisome in relatively low doses was effective in reducing dissemination of pulmonary aspergillosis we suggest that these doses may have a place in prophylactic settings. The same may be true for early empiric therapy, and other investigators recently showed that in neutropenic patients with fever of unknown origin (FUO), the time to resolution of symptoms did not significantly differ between patients receiving higher (3 mg/kg) and lower (1 mg/kg) doses of AmBisome although it is very difficult to draw conclusions as to antifungal efficacy from a FUO study.[48]

To compare the efficacy of higher dosages of AmBisome to that of AmB in a clinical setting, we conducted two randomized comparative multicenter studies (chapter 7 and 9). In HIV-infected patients with cryptococcal meningitis, we showed that patients treated with AmBisome had an earlier CSF-culture conversion (better mycological response) than patients treated with AmB and that the time to CSF-culture conversion was significantly related to the time to clinical response. Although in this study, AmB was clinically as effective as AmBisome, these findings led to the assumption that a better clinical response rate might have been found when more patients had been studied. A better mycological response was not found by other investigators who studied several doses of Abelcet (1.2 to 5.0 mg/kg) against AmB for cryptococcal meningitis in HIV-infected patients, which underscores the fact that the results obtained with one of the lipid-based formulations can not be extrapolated to the other formulations.[49] The same can be concluded from a recent study comparing all three lipid formulations and AmB for the treatment of systemic cryptococcosis in a mouse-

model.[50] In this study high doses of AmBisome and ABCD were more effective than high doses of ABLC which was more effective than AmB. Other investigators have shown that the addition of 5FC to AmB as initial therapy for cryptococcal meningitis in HIV-infected patients was beneficial in terms of reducing the number of relapses.[51] Relapses reported by these investigators who used two weeks of AmB might also have been due to a too early switch to azole therapy since no relapses were noted if patients initially had received three weeks of AmB or AmBisome in our study. When initial therapy is too short, it is possible that too many viable cryptococci remain, which are able to reproduce when therapy with AmB is substituted by azole therapy.

AmBisome had superior clinical efficacy compared to AmB in patients with neutropenia-associated fungal infections. Although the majority of fungal infections in this study could not be documented microbiologically, this deemed not to devaluate the results because in clinical practice these are the exact patients that are given antifungal therapy. In addition, patients with other documented microbiological diagnoses were excluded from the analysis. AmBisome showed a significantly higher response rate at 14 days and more patients had a complete response at termination of therapy. We stated that a complete response offers better opportunities for continuing treatment of the underlying malignancy with new courses of cytotoxic chemotherapy. However, even in patients that have recovered from invasive aspergillosis, the risk of relapse of fungal disease is estimated to be approximately 50%. This underscores the importance of mycological eradication by effective antifungals. As in patients with cryptococcal meningitis, AmBisome seemed to result in a better mycological outcome in patients with neutropenia-associated fungal infections. A longer follow-up of patients treated with this formulation should answer the question whether it reduces the number of relapses during subsequent cytotoxic chemotherapy as compared to patients treated with AmB. AmBisome significantly reduced mortality in patients with progressive malignancy treated for invasive fungal infections and the status of the underlying malignancy was the most important independent factor predictive of outcome independent of the duration of neutropenia. Probably patients with progressive malignancy have the fewest properly functioning neutrophils, and successful treatment depends almost exclusively on the antifungal agents used. Therefore, an agent with maximal mycological efficacy should be given. This might also be true for certain other patients, for example bone-marrow and peripheral-stamcell transplant patients. We recommend that future studies document the malignancy status in order to correct for this determinator

when comparing the results of different studies. Furthermore, patients should be stratified for this factor in future randomized trials.

Recently some other studies have shown lipid-based products of AmB to be efficacious in clinical settings.[52,53] Some of these studies have compared results of patients treated with the newer lipid-based formulations to historical controls treated with AmB.[53] Such study design seems not to be justified because other empirical regimens, improved diagnostic methods including automated blood-culture systems, CT-scans, ELISA and PCR and increased clinical awareness have probably resulted patients with invasive fungal infections being diagnosed at an earlier stage of their disease. Thus, these factors may have contributed to an overall better outcome when compared to historical controls.

In addition to the commercial products, the efforts of French investigators, who produced an emulsion at the bedside by mixing intralipid 20% with AmB, have to be mentioned.[54,55] Doubt about its efficacy and its pharmacological instability would suggest that this mixture may not have much clinical value.[56] The studies with this mixture probably were initiated because of the high costs of the lipid-based formulations of AmB marketed by the pharmaceutical industry.[57,58] However, our studies suggest that therapy with AmBisome may become more cost-effective. First, in case of HIV-infected patients with cryptococcal meningitis the duration of hospital stay can be reduced because initial therapy can be shortened and treatment on an out-patient basis is possible (chapter 9). Secondly, low toxicity rates, the administration AmBisome through peripheral veins and shorter infusion times all reduce secondary costs (chapter 7 and chapter 9). Finally, in certain groups of patients, AmBisome reduces the mortality from fungal infection (chapter 7). Therefore, we recommend that future comparative studies between lipid-based products of AmB and AmB, should include a formal cost benefit analysis for the use of these products. Furthermore, because optimal doses are not determined yet, and overall results from animal models show that higher doses are more effective, clinical studies should investigate the role of even higher doses of AmBisome than have been used until now. Toxicity data from the two clinical trial we performed, clearly show that the doses used were well below the maximal tolerated doses. This also seems to be true for the other lipid formulations which in future studies should be compared to each other for efficacy and toxicity in several doses. Although the number of opportunistic fungal infections is increasing, the number of these infections in a single center will remain limited, and studies as mentioned above can thus only be conducted in a multicenter effort.

Conclusions

The studies presented in this thesis show that epidemiological research in the field of invasive fungal infections can be simplified by genotyping strains, e.g. by RAPD. Genotyping helped to elucidate some questions of the complex epidemiology of fungal infection and this will add to the management of these infections. The animal and clinical studies with AmBisome showed that this formulation has a place in the treatment of invasive fungal infections in neutropenic patients and of cryptococcal meningitis in HIV-infected patients. However, many questions remain with regard to the management of invasive fungal infections. Epidemiological studies should address to the question whether further barrier/isolation procedures are beneficial. Randomized comparative trials should seek for effective prophylactic regimes, for which low dose AmBisome is a candidate. Optimalisation of diagnostic procedures is necessary to enable pre-emptive therapy and comparative trials should determine optimal treatment regimens for this purpose. Optimalisation of sensitivity testing for both yeasts and molds is warranted such that we are able to detect resistant strains and to guide antifungal therapy. Randomized comparative trials have to determine optimal therapy when despite all, full blown invasive fungal infection has developed in an immunocompromised patient. High doses of liposomal AmB should be studied in this setting. Finally, the place of other novel antifungals should be established both in animal and in clinical studies.[33,59-61]

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Summary and samenvatting

SUMMARY

The incidence of opportunistic fungal infections has been increasing the past three decades along with the increasing number of susceptible patients. Factors responsible for this development are the extensive use of antibacterial agents, the use of cytotoxic chemotherapy, organ transplantation combined with the use of immunosuppressive therapy, the use of indwelling catheters and the pandemic caused by the human immunodeficiency virus (HIV). However, other developments in medicine may bring some relieve from this trend. The new anti-retroviral treatment regimens have been shown to decrease the incidence of opportunistic infections in HIV-infected patients. Alterations in immunosuppressive therapy may soon follow. All these developments taken together have pushed opportunistic fungal infections into the category of emerging infectious diseases that urgently require more attention. However, safe and highly effective antifungal agents are still not available today. The ongoing search for new, more active and less toxic drugs has resulted in the development of new agents and new (lipid-based) formulations of older agents. The exact place of these new agents and formulations has not yet been established, but none of them seems to be the magic bullet against fungi. Therefore, prevention of invasive fungal infections in immunocompromised patients remains an extremely important strategy and studies are conducted to learn about the epidemiology of these infections to develop appropriate preventive strategies.

The **aim of the studies** presented in this thesis was to gain further insight in the epidemiology of opportunistic fungal infections and to compare the toxicity and efficacy of one of the lipid-based formulations of amphotericin B (AmB), namely AmBisome, to that of AmB in the treatment of these infections.

Epidemiology (chapters 2 to 4)

To gain further insight in the epidemiology of nosocomial invasive aspergillosis, an apparent outbreak of invasive aspergillosis at our hematology department was investigated in depth (chapter 2), and a surveillance program for fungal conidia inside the hospital and in the outside air was conducted thereafter (chapter 3). The initial question while investigating an outbreak of nosocomial invasive aspergillosis is whether it is due to a fungal source within the department in question or to an increased introduction of fungi into this area. We tried to answer this question by fingerprinting clinical and environmental fungal isolates by means of randomly amplified polymorphic DNA (RAPD). We showed the

importance of using the right type and number of RAPD-primers to obtain satisfactory discriminative power, when this technique is applied for the genotyping of fungal isolates (chapter 2). Although no exact cause could be determined for the apparent outbreak of invasive aspergillosis, we showed that fingerprinting of fungal isolates was helpful to focus further investigations. From the fingerprinting patterns of clinical and environmental isolates, it became clear that the outbreak was probably caused by the introduction of conidia from sources elsewhere either within or outside the hospital. Further investigations were therefore focussed on leakages in barrier systems and proper functioning of the air-conditioning systems.

Genotyping of environmental isolates obtained during surveillance of the air in the department of hematology showed that clones of *Aspergillus fumigatus* were able to remain present at this department for periods of more than a year (chapter 3). Because almost all environmental isolates from this department belonged to one of the persisting clones, and because they were all genotypically different from the patients isolates, we suggested that these patients were probably infected with strains not derived from the hospital air. This finding could have major consequences in the development of strategies to prevent invasive aspergillosis.

Results from our surveillance study further showed that the measurement of conidia of non-pathogenic fungi, which are present in 10-fold higher densities, can be taken as sensitive parameter in the control of barrier systems (chapter 3).

In a molecular typing study of cryptococci we showed that RAPD can be used for the typing of other fungal pathogens as well and that the results match the results of other (geno-)typing methods (chapter 4). The RAPD patterns of *Cryptococcus neoformans* from HIV-patients with cryptococcal meningitis treated in Rotterdam showed less variation when compared to the patterns obtained with *Aspergillus* spp. (chapter 2). Because pulsed-field gel electrophoresis (PFGE) resulted in the same variation it was suggested that clonal expansion of *C. neoformans* has occurred in this restricted region.

Management (chapters 5 to 9)

Even today the number of drugs that can effectively be used for the treatment of invasive fungal infections in immunocompromised patients remains limited. The most widely used drug (AmB) has major side effects and relatively high failure rates, which underscores the importance of developing new antifungal drugs and formulations. This has resulted in the development of the relatively non-toxic

triazoles (fluconazole and itraconazole). However, these agents also have some disadvantages; azoles are said to be fungistatic, fluconazole is not active against moulds, itraconazole is only available as oral formulation and, probably most importantly, azole-resistent fungal pathogens have "emerged". Because of the aforementioned, AmB remains the therapy of choice for many fungal infections especially in immunocompromised patients. To overcome problems with its toxicity, lipid-based formulations of AmB have been developed. Three such formulations have been marketed: Amphotericin B Lipid Complex (ABLC, Abelcet), Amphotericin B Colloidal Dispersion (Amphocil) and liposomal amphotericin B (AmBisome). We reviewed the literature on the use of these formulations in animal models and in clinical settings in chapter 5. This review makes clear that the three formulations not only differ significantly in their composition and pharmacokinetics, but probably also in efficacies for the treatment of several infections.

To compare the efficacy of AmBisome to that of AmB we developed a rat-model of invasive pulmonary aspergillosis that mimicked human disease during neutropenia; one-sided onset of infection of the lung, persistent granulocytopenia and start of treatment at a time when hyphal growth is firmly established (chapter 6). In this model, no differences in efficacy in mortality were found between high doses of AmBisome and AmB. Lower doses of AmBisome (equal to AmB) were not able to reduce mortality as compared to untreated animals. However, compared to AmB, both high and low doses of AmBisome significantly diminished dissemination of infection to the other lung and other organs. Most other animal-models comparing lipid formulations of AmB with AmB show comparable results, with the highest doses being most effective and doses equal to the optimal dose of AmB being less effective than AmB (chapter 5). Because we showed a reduction of dissemination of infection also when low doses of AmBisome were used, we suggested to study the use of these doses in a prophylactic setting.

To compare the efficacy of "high" doses of AmBisome to that of AmB in a clinical setting, we conducted two randomized comparative multicenter studies (chapter 7 and 9).

In the first study in patients with neutropenia-associated fungal infections we found that AmBisome had superior clinical efficacy compared to AmB. Although the majority of fungal infections in this study could not be documented microbiologically, this did not devaluate the results because in clinical practice these are the patients that are given antifungal therapy, and in addition, patients with other documented microbiological diagnoses were excluded from the analy-

sis. AmBisome showed a significantly higher response rate at 14 days and more patients had a complete response at termination of therapy. We stated that a complete response offers better opportunities for continuing treatment of the underlying malignancy with new courses of cytotoxic chemotherapy. In patients with a microbiologically documented infection in this same study, AmBisome seemed to result in a better mycological outcome. The most important result of this study was that we could show that AmBisome significantly reduced mortality in patients with progressive malignancy treated for invasive fungal infections. The status of the underlying malignancy was the most important independent factor predictive of outcome. Probably patients with progressive malignancy have the fewest properly functioning neutrophils, and successful treatment depends almost exclusively on the antifungal agents used. Therefore, an agent with maximal mycological efficacy should be given. We recommend from these results that future studies document the malignancy status in order to correct for this determinator when comparing the results of different studies. Furthermore, patients should be stratified for this factor in future randomized trials.

Before we studied the efficacy of AmBisome for the treatment of cryptococcal meningitis in HIV-infected patients, we looked retrospectively for factors predictive of outcome of this infection (chapter 8). We showed that patients with a lumbar opening pressure > 30 cm H_2O at diagnosis had a significantly higher chance of a complicated course of infection as compared to patients who had a lumbar opening pressure < 30 cm H_2O. This observation was confirmed in the prospective comparable trial in HIV-infected patients with cryptococcal meningitis (chapter 9). In this study we showed that patients treated with AmBisome had an earlier cerebrospinal fluid (CSF)-culture conversion (better mycological response) than patients treated with AmB and that the time to CSF-culture conversion was significantly related to the time to clinical response. Although in this study, AmB was clinically as effective as AmBisome, these findings led to the assumption that a better clinical response rate for AmBisome might have been found when more patients had been studied.

Besides the differences in efficacy, in both clinical studies we clearly showed that nephrotoxicity with the use of AmBisome was significantly diminished as compared to the use of AmB. No (new) major adverse events with the use of AmBisome were encountered.

The studies presented in the first part of this thesis show that epidemiological research in the field of invasive fungal infections can be simplified by genotyping

fungal strains, e.g. by RAPD. The results of genotyping can focus investigations of outbreaks of nosocomial invasive aspergillosis in a certain direction. Genotyping also helped us to elucidate some questions of the complex epidemiology of fungal infection and this will add to the management of these infections.
In the second part, animal and clinical studies with AmBisome showed that, although optimal doses still have to be determined, this formulation seems to have a place in the treatment of invasive fungal infections in neutropenic patients and of cryptococcal meningitis in HIV-infected patients.

SAMENVATTING

De incidentie van opportunistische fungale infecties is de laatste dertig jaar fors toegenomen parallel aan een toename van het aantal vatbare patiënten. Een aantal factoren zijn verantwoordelijk voor deze ontwikkeling: het uitgebreide gebruik van antibacteriele middelen, het gebruik van cytotoxische chemotherapie, orgaan transplantaties gepaard met immuunsuppressieve therapie, het gebruik van vasculaire lijnen en de pandemie veroorzaakt door het human immunodeficiency virus (HIV). Al deze factoren zorgen voor beschadiging van een of meer afweer-linies tegen fungale infecties. Andere ontwikkelingen in de geneeskunde echter zouden deze trend wat kunnen ombuigen. De nieuwe anti-retrovirale schema's hebben gezorgd voor een daling van het aantal opportunistische infecties in HIV-geïnfecteerde patiënten en mogelijk volgen binnenkort aanpassingen van immuunsuppressieve therapie. Al deze ontwikkelingen hebben er voor gezorgd dat opportunistische fungale infecties terecht zijn gekomen in de categorie van de "emerging" infecties. Desondanks zijn veilige en goed effectieve antifungale middelen nog steeds niet beschikbaar. De voortgaande zoektocht naar nieuwe, meer effectieve en minder toxische middelen heeft geresulteerd in de ontwikkeling van geheel nieuwe middelen en van nieuwe (lipid) formuleringen van oude middelen. De exacte plaats van deze middelen is nog niet bepaald, maar duidelijk lijkt dat geen van deze middelen de "magic bullet" tegen fungi zal zijn. Daarom blijft preventie van invasieve fungale infecties in immuungecompromiteerde patiënten uiterst belangrijk en worden studies verricht om meer te weten te komen over de epidemiologie van deze infecties om passende preventieve maatregelen te bedenken.

Het **doel van de studies** gepresenteerd in dit proefschrift was om meer inzicht te krijgen in de epidemiologie van opportunistische fungale infecties en om de toxiciteit en effectiviteit van een van de lipid formuleringen van amfotericine B (AmB), namelijk AmBisome, te vergelijken met die van AmB zelf, voor de behandeling van deze infecties.

Epidemiologie (hoofdstukken 2 tot 4)

Om meer inzicht te krijgen in de epidemiologie van nosocomiale invasieve aspergillose werd een mogelijke uitbraak van invasieve aspergillose op de afdeling hematologie intensief bestudeerd (hoofdstuk 2), en werd een surveillance programma opgestart voor schimmelsporen in de lucht in en buiten het

ziekenhuis (hoofdstuk 3). De eerste vraag die moet worden beantwoord wanneer een uitbraak van nosocomiale invasieve aspergillose wordt onderzocht is of deze het gevolg is van een bron op de afdeling zelf, of het gevolg is van een toegenomen introductie van sporen van buitenaf. Wij probeerden deze vraag te beantwoorden door het "fingerprinten" van klinische en omgevings-isolaten van schimmels, door middel van randomly amplified polymorphic DNA (RAPD). We toonden het belang aan van het gebruik van het juiste type en het juiste aantal RAPD-primers om voldoende onderscheidingsvermogen te krijgen wanneer deze techniek wordt gebruikt voor het genotyperen van schimmelisolaten (hoofdstuk 2). Hoewel geen precieze oorzaak kon worden aangetoond voor de uitbraak van invasieve aspergillose, toonden we aan dat het fingerprinten van schimmelisolaten hielp om de onderzoekingen in een bepaalde richting te sturen. Uit de fingerprint-patronen van de klinische en omgevings-isolaten werd duidelijk dat de uitbraak waarschijnlijk was veroorzaakt door een toegenomen introductie van schimmelsporen vanuit een andere plaats binnen of buiten het ziekenhuis. Verder onderzoek werd daarom gefocust op lekken in de barrières en het goed functioneren van de air-conditioning systemen.

Genotypering van omgevings-isolaten die werden verzameld gedurende de surveillance van lucht van de afdeling hematologie toonde aan dat bepaalde kloons van *Aspergillus fumigatus* in staat waren om > 12 maanden op deze afdeling aanwezig te blijven (hoofdstuk 3). Omdat bijna alle isolaten uit de lucht van deze afdeling tot een van deze persisterende kloons behoorde en omdat deze genotypisch verschilden van isolaten van patiënten, suggereren wij dat deze patiënten geïnfecteerd werden met stammen niet afkomstig uit het ziekenhuis. Deze bevinding kan consequenties hebben voor de ontwikkeling van strategieën ter preventie van invasieve aspergillose.

Resultaten van onze surveillance studie toonden verder aan dat het meten van sporen van niet-pathogene schimmels, welke in 10-voudig hogere concentraties in de lucht aanwezig zijn, kan worden gebruikt als een gevoelige parameter voor de controle van barrière systemen (hoofdstuk 3).

In een moleculaire typering studie van cryptococcen toonden we aan dat RAPD ook gebruikt kan worden voor de genotypering van andere fungale pathogenen en dat de resultaten van RAPD goed overeen komen met de resultaten van andere (geno-)typerings methodes (hoofdstuk 4). De RAPD-patronen van isolaten van *Cryptococcus neoformans* van HIV-patiënten met cryptococcen meningitis behandeld in Rotterdam toonden minder variatie dan de RAPD-patronen van *Aspergillus* spp. (hoofdstuk 2). Het feit dat de pulsed-field

gel electrophoresis (PFGE)-patronen van deze isolaten dezelfde geringe variatie vertoonden suggereert dat klonale expansie van *C. neoformans* heeft plaatsgevonden in deze omschreven regio.

Management (hoofdstukken 5 tot 9)

Zelfs op dit moment is het aantal effectieve middelen dat gebruikt kan worden voor de behandeling van invasieve fungale infecties bij immuungecompromitteerde patiënten beperkt. Het meest gebruikte middel (AmB) heeft belangrijke bijwerkingen en therapie faalt in een relatief hoog aantal gevallen. Dit onderstreept het belang van het ontwikkelen van nieuwe antifungale middelen en formuleringen. Dit heeft geresulteerd in de ontwikkeling van de relatief weinig toxische tri-azolen (fluconazole en itraconazole). Echter ook deze middelen hebben een aantal nadelen; azolen worden beschouwd als fungistatisch, fluconazole is niet actief tegen schimmels, itraconazole is alleen beschikbaar als orale formulering en, waarschijnlijk het belangrijkst, azole-resistente fungi zijn "opgedoken". Vanwege het bovenstaande blijft AmB het middel van eerste keuze voor de behandeling van vele fungale infecties, met name in immuungecompromiteerde patiënten. Om het probleem van de toxiciteit van AmB te overwinnen zijn lipidformuleringen van AmB ontwikkeld. Drie van deze formuleringen zijn inmiddels commercieel verkrijgbaar: Amphotericin B Lipid Complex (ABLC, Abelcet), Amphotericin B Colloidal Dispersion (Amphocil) en liposomaal amphotericin B (AmBisome). Wij reviewden de literatuur betreffende het gebruik van deze formuleringen in dier-modellen en in klinische studies (hoofdstuk 5). Deze review maakte duidelijk dat de drie formuleringen niet alleen behoorlijk verschillen in samenstelling en farmacokinetiek, maar waarschijnlijk ook in effectiviteit met betrekking tot de behandeling van verschillende fungale infecties.

Om de effectiviteit van AmBisome te vergelijken met die van AmB ontwikkelden we een ratten-model voor invasieve pulmonale aspergillose dat de infectie bij de mens tijdens neutropenie sterk nabootst; eenzijdig begin van de infectie, persisterende granulocytopenie en het starten van behandeling nadat hyfen-groei is vastgesteld (hoofdstuk 6). In dit model werden geen verschillen gevonden voor wat betreft mortaliteit tussen dieren behandeld met hoge doses AmBisome of met AmB. Lagere doses AmBisome (gelijk aan de AmB-dosis) hadden geen invloed op mortaliteit ten opzichte van onbehandelde dieren. Echter in vergelijking met AmB, verminderden zowel hoge als ook lage doses AmBisome de disseminatie van de infectie naar de andere long en naar andere organen. In de meeste andere studies die lipid formuleringen van AmB met AmB vergelijken in dier-

modellen worden vergelijkbare resultaten gevonden; hoge doses van de lipid-formuleringen zijn het meest effectief, terwijl doses vergelijkbaar met AmB doses minder effectief zijn dan AmB zelf (hoofdstuk 5). Omdat wij in ons ratten-model aangetoond hebben dat lage doses AmBisome een vermindering geven van disseminatie van de infectie, stellen wij voor dat deze doses bestudeerd gaan worden voor profylactisch gebruik.

Om de effectiviteit van "hoge" doses AmBisome te vergelijken met AmB in een klinische setting werden twee gerandomiseerde vergelijkende multicenter studies uitgevoerd (hoofdstukken 7 en 9).

In een studie bij patiënten met neutropenie-geassocieerde fungale infecties vonden we dat AmBisome klinisch effectiever was dan AmB. Hoewel het merendeel van de fungale infecties niet microbiologisch kon worden gedocumenteerd, vonden wij niet dat dit onze resultaten devalueerde omdat in de kliniek dit ook juist de patiënten zijn die behandeld worden met antifungale therapie; bovendien werden patiënten bij wie een andere oorzaak voor de verschijnselen werd gevonden, van de studie uitgesloten. Het response percentage op dag 14 van patiënten behandeld met AmBisome was significant hoger dan dat van patiënten behandeld met AmB en meer patiënten vertoonden een volledige response bij het beëindigen van de therapie. Wij denken dat een volledige response betere kansen biedt om door te gaan met de behandeling van de onderliggende maligniteit door middel van het geven van nieuwe cytotoxische chemokuren. Van patiënten met een microbiologisch bewezen infectie in deze zelfde studie, leken zij die behandeld werden met AmBisome een betere mycologische respons te vertonen. Het belangrijkste resultaat van deze studie was echter dat we konden aantonen dat behandeling met AmBisome resulteerde in een significante daling van de mortaliteit onder patiënten met progressieve maligniteit. De status van de maligniteit was de belangrijkste onafhankelijk voorspellende factor voor outcome. Waarschijnlijk hebben patiënten met progressieve maligniteit het minste aantal goed functionerende neutrofiele granulocyten en is succesvolle behandeling van de fungale infectie bijna geheel afhankelijk van het gebruikte middel. Daarom is het belangrijk een middel te geven met maximale mycologische effectiviteit. Wij raden aan om in toekomstige studies de status van de onderliggende maligniteit te documenteren zodat bevindingen gecorrigeerd kunnen worden voor deze factor wanneer resultaten van studies onderling worden vergeleken. Verder zouden patiënten voor deze factor gestratificeerd dienen te worden.

Voordat we de effectiviteit van AmBisome bestudeerden in de behandeling van cryptococcen meningitis bij HIV-geïnfecteerde patiënten, zochten we eerst

retrospectief naar factoren die voorspellend waren voor het beloop van deze infectie (hoofdstuk 8). We konden aantonen dat patiënten met een lumbale openingsdruk > 30 cm H_2O bij diagnose een significant hogere kans hadden op een gecompliceerd beloop van de infectie in vergelijking met patiënten met een lumbale openingsdruk < 30 cm H_2O. Deze bevinding werd bevestigd in de prospectieve vergelijkende studie in HIV-geïnfecteerde patiënten met cryptococcen meningitis (hoofdstuk 9). In deze studie toonden we aan dat patiënten behandeld met AmBisome een significant snellere conversie van hun liquor-kweek (betere mycologische response) hadden in vergelijking met patiënten behandeld met AmB. Bovendien was de tijd tot liquor-kweek conversie significant gerelateerd aan de tijd tot klinische respons. Hoewel in deze studie AmB klinische net zo effectief was als AmBisome, leidden deze bevindingen tot de veronderstelling dat een betere klinische response na behandeling met AmBisome zou zijn gevonden indien meer patiënten waren bestudeerd.

De studies gepresenteerd in de eerste hoofdstukken van dit proefschrift tonen aan dat epidemiologisch onderzoek op het gebied van invasieve schimmel infecties vereenvoudigd kan worden door middel van het genotyperen van schimmel isolaten bijvoorbeeld door middel van RAPD. De resultaten van deze genotypering kunnen helpen om de richting van het onderzoek naar de oorzaak van een uitbraak van nosocomiale invasieve aspergillose te bepalen. Verder heeft genotypering van schimmel isolaten ons geholpen bij het verhelderen van een aantal aspecten van de complexe epidemiologie van fungale infecties en dit komt weer ten goede aan de aanpak van deze infecties.
In het tweede deel van het proefschrift hebben wij door middel van studies in een diermodel en twee klinische studies aangetoond dat AmBisome, ondanks het feit dat nog steeds geen optimale dosis van het middel bepaald is, een plaats lijkt te hebben in de behandeling van invasieve schimmel infecties in neutropene patiënten en van cryptococcen meningitis bij HIV-geïnfecteerd patiënten.

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CURRICULUM VITAE

Sander Leenders werd op 3 juli 1965 geboren te Eindhoven. In 1983 behaalde hij het diploma Atheneum B aan het Sint Joriscollege te Eindhoven. In datzelfde jaar begon hij de studie geneeskunde aan de Rijksuniversiteit te Utrecht. Daar werd in 1988 het doctoraalexamen behaald en vervolgens in 1991 het arts-examen. Van 1991 tot 1992 was hij in het kader van de dienstplicht werkzaam als Luitenant-arts bij de Koninklijke Landmacht (OCMT te Hollandsche Rading). Van 1992 tot 1994 was hij aangesteld op de afdeling medische microbiologie en infectieziekten van het Academische Ziekenhuis Rotterdam-Dijkzigt eerst als algemeen arts, later als AGNIO, en werd aangevangen met het onderzoek dat tot het huidige proefschrift leidde. Van 1994 tot november 1998 was hij in opleiding tot medisch microbioloog in het Academisch Ziekenhuis Rotterdam (opleider: Prof. Dr. H.A. Verbrugh). Sedert 1 november 1998 is hij ingeschreven als arts-microbioloog in het specialisten register. Per januari 1999 is hij als arts-microbioloog werkzaam in het Bosch Medicentrum te 's-Hertogenbosch.
Sinds 1994 is hij getrouwd met Marjan Wassenaar. In 1998 werd hun dochter Meilinde geboren.