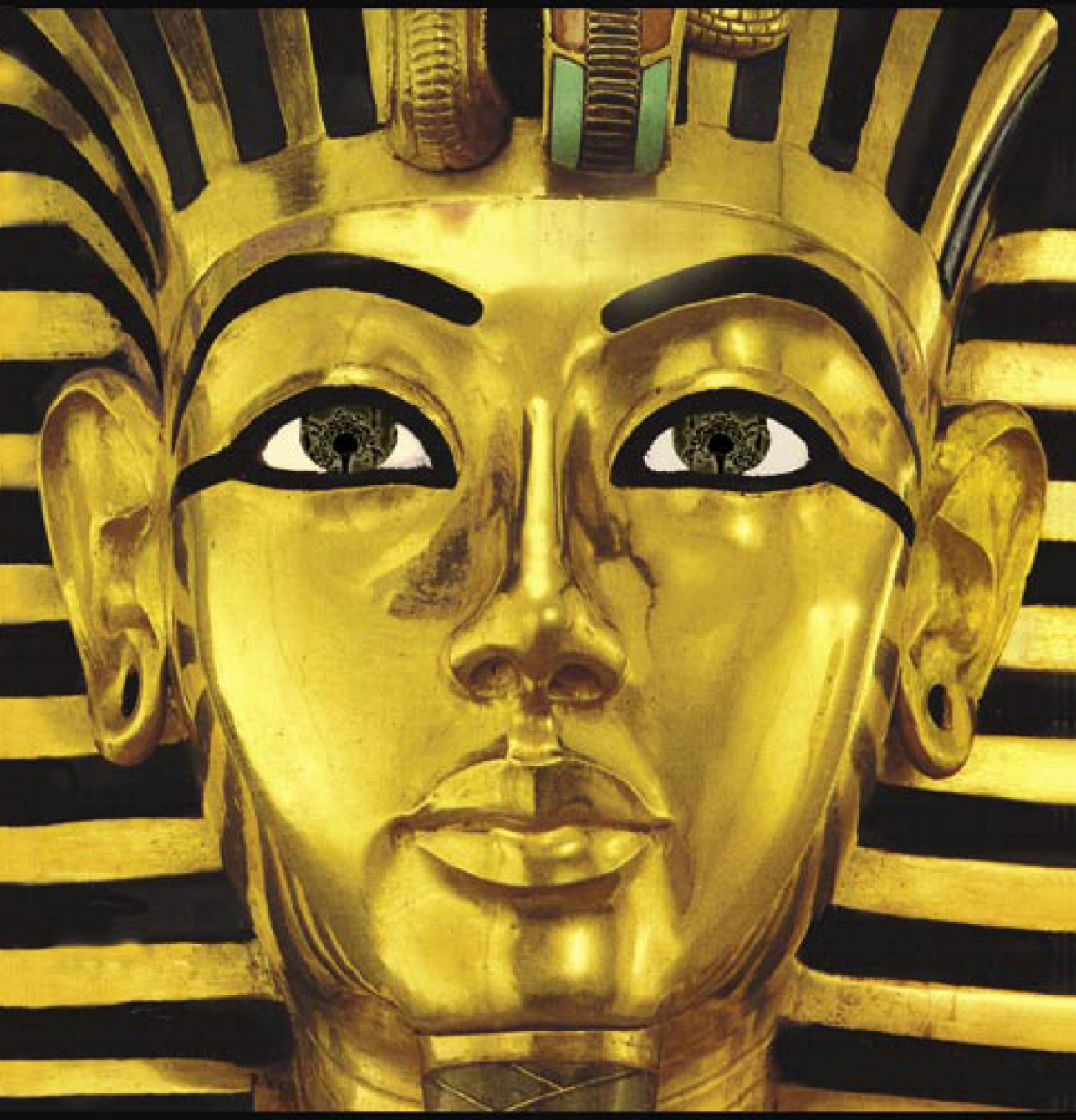
Experimental and clinical studies on invasive pulmonary aspergillosis: pathophysiology, diagnosis and management



Martin J. Becker

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Voor Nurma en Thomas

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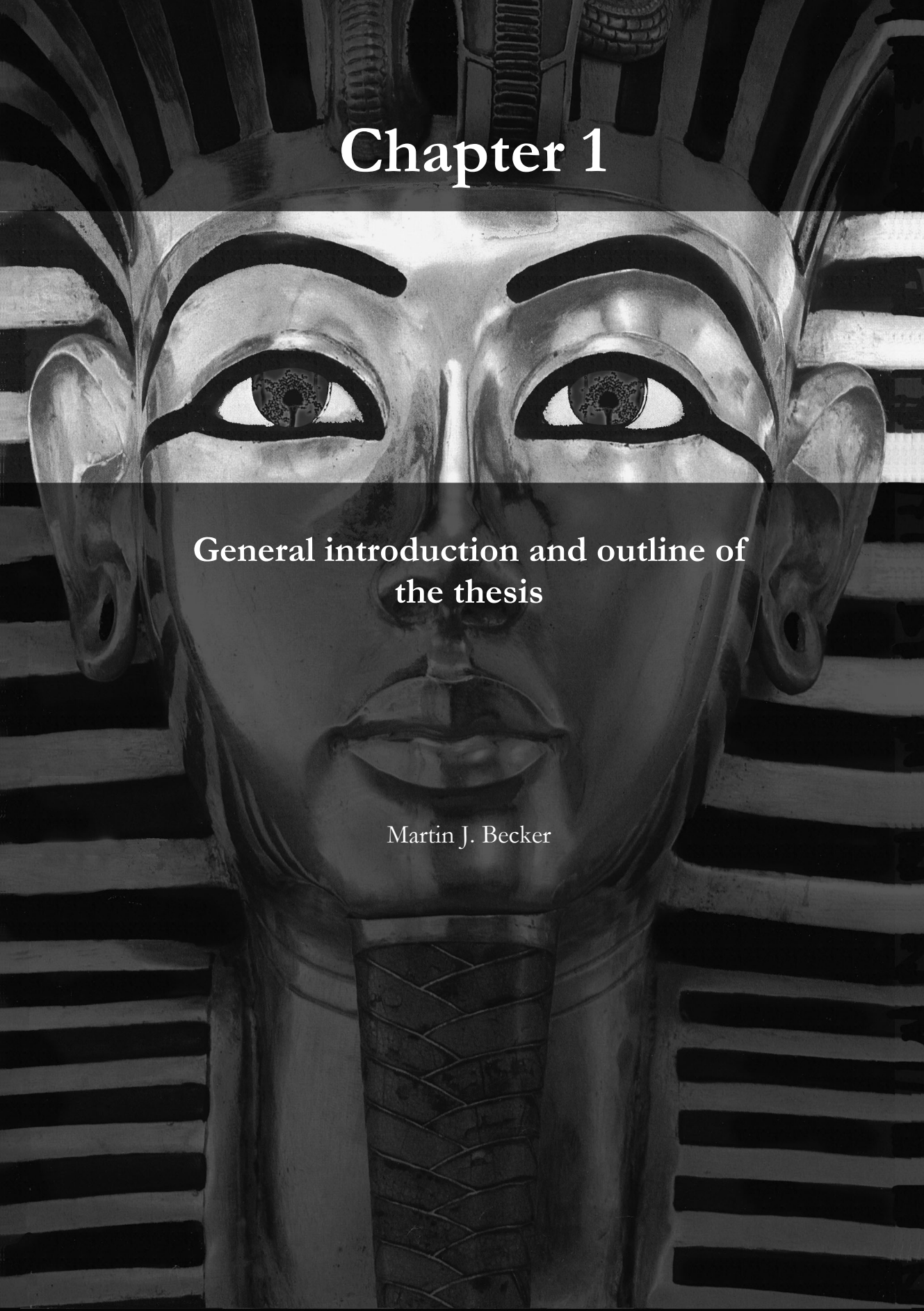

Chapter 1

General introduction and outline of the thesis

Martin J. Becker

INVASIVE PULMONARY ASPERGILLOSIS, EPIDEMIOLOGY AND PATIENT GROUPS

Aspergillus is a saprofytic fungus that grows in humid environments on decaying organic matter. Its ability to adapt to a wide variability of conditions accounts for its worldwide distribution. Invasive aspergillosis (IA) is a disease characterised by invasion of normal tissue by *Aspergillus* organisms, resulting in significant tissue damage and necrosis. Most commonly, IA develops in patients with impaired host defence. Factors that predispose patients to develop IA include prolonged granulocytopenia, the development of graft-versus-host disease, immunosuppressive therapy, the use of adrenal corticosteroids, and the prolonged reduction of host defences associated with diseases such as chronic granulomatous disease [1].
The most common route of infection is the inhalation of fungal spores, and therefore the majority of IA patients (± 90%) develop an invasive pulmonary aspergillosis (IPA). Other manifestations of the disease include IA of the paranasal sinuses, skin, wounds and eyes [2].

There are several factors that make IPA a challenging fungal infection to manage:

- IPA is currently a major direct or contributory cause of death in severely immunocompromised patients such as haemato-oncological patients receiving chemotherapy [3], and its incidence has shown a significant increase in the past few decades [4,5].
- IPA is difficult to diagnose, especially in the early stages of the disease.
- The treatment of IPA is suboptimal, with reported response rates below 55% in leukaemia- and bone marrow transplant patients. [6].

PATHOGENESIS OF INVASIVE PULMONARY ASPERGILLOSIS

Pathology

Exposure to *Aspergillus* occurs via inhalation of the ubiquitous conidia, which are small enough (diameter 3 to 5 μm) to reach the alveoli. The most important determinant of infection is the immune status of the patient, not the intensity of exposure [7]. In neutropenic patients, *Aspergillus* can invade the lung through the alveoli or the tracheobronchial tree. Invasion through the submucosa, into the cartilage, and into contiguous blood vessels is common. This invasive growth leads to hemorrhagic infarction and necrosis [8]. Neutrophils are seen in lesions of neutropenic patients whose bone marrow function has returned, as well as in tissues of less neutropenic patients. The pulmonary infiltrates may be single or multiple and tend to be enlarging, rounded infiltrates, often involving a large proportion of the lung. Grossly, the involved lung has the appearance of an infarct, being dark red and very firm, often with concentric rings of haemorrhage and necrosis with poor demarcation at the advancing

front of the infarct. Intra-pulmonary hematogenous dissemination, occurring in less than 10% of cases, produces myriads of small target lesions that are referred to as miliary micro-abscesses [9]. Extra-pulmonary hematogenous dissemination occurs in 25-35% of patients with IPA, affecting especially the central nervous system, and less commonly liver, skin, kidneys, pleura, heart, and other sites [4,10].

Host immune response

As stated above, the respiratory tract is the portal of entry in human IPA. It is postulated that innate immunity is the principal pathway by which *Aspergillus* is cleared from the lung. This innate response consists of alveolar macrophages, which represent the first line of defence against conidia entering the lung, and recruited neutrophils. Neutrophils are believed to kill conidia that have survived to form hyphae and cause invasive infection [11]. Various cells of the monocytic lineage can engulf and kill *Aspergillus* conidia in vitro [12,13] and *in vivo* [14,15]. Both macrophages [16] and neutrophils [11,17] have been shown to damage hyphae *in vitro*.

Both cyto-and chemokines may play an important role in the pathogenesis of IPA. In murine studies on IA, production of Th1 and Th2 cytokines occurred differently in mice resisting or succumbing to the infection. Development of protective acquired immunity was associated with the activation of CD4+ Th1 cells producing IFNγ and macrophages producing IL12. In contrast, production of IL4 and IL10 by $CD4^+$ Th2 cells was associated with disease progression [18]. In addition, the production of the pro-inflammatory cytokine TNFα was associated with survival in several studies [19,20]. Chemokines also seem to play a role in IPA. Two in vivo studies have demonstrated that intra-tracheal inoculation of mice with *Aspergillus fumigatus* results in an increased production of the chemokines MCP-1, MIP-1α and MIP-2 by broncho-alveolar lavage (BAL) cells or as measured in whole lung homogenates [21,22].

DIAGNOSIS

Clinical signs

Clinical signs of IPA are often non-specific. Cough, usually unproductive, and fever are the most frequent presenting symptoms in neutropenic patients. Two other symptoms that are significant and that raise the possibility of IPA in the appropriate clinical setting are pleuritic chest pain (due to small pulmonary infarctions secondary to vascular invasion) and haemoptysis that is usually mild but could be massive. However, these symptoms are only present in a proportion of patients. Other symptoms of IPA may be due to dissemination of the fungus to extra-pulmonary organs, leading to corresponding symptoms [4] .

Radiological signs

The chest radiograph often shows non-specific changes. Rounded densities, pleural-based infiltrates that are suggestive of pulmonary infarctions, and cavitation are only seen in a proportion of patients on the chest radiograph [23,24] . In contrast, CT scanning of the chest has had a major impact on the management of patients with IPA [25,26]. It is more sensitive and specific than chest radiography [25] and is particularly valuable when chest X ray is negative or shows only subtle changes. Typical chest CT scan findings in patients with IPA are multiple nodules, the "halo"- sign, which is an early radiological sign that appears as a zone of low attenuation due to haemorrhage surrounding the pulmonary nodule [27], and the "air crescent" sign, which is a crescent shaped lucency, formed after retraction of a central necrotic mass. In addition, cavitations also suggest an invasive fungal infection [28]. Whereas "halo"-signs are often seen in the early course of the disease, the "crescent" – sign and cavitation usually correlate with recovery from neutropenia and are relatively late findings [29]. These three radiological signs are relatively specific for IPA, but they are neither sensitive nor pathognomonic, since other fungal infections and also some non-fungal conditions may have a similar appearance [27,28].

Histopathology and culture

The only definite method to diagnose IPA is to demonstrate the presence of septate, acute, branching hyphae in a lung tissue sample in combination with a culture that is positive for *Aspergillus* from the same site. However, large biopsies are often precluded by trombocytopenia or by the critical condition of the patient, whereas the yield of transthoracic fine needle aspirates is often disappointing.

With regard to fungal culture, studies have shown that sputum- and BAL-fluid samples that are positive for *Aspergillus* in patients with leukaemia or in those who have undergone BMT have a positive predictive value of 80 to 90% [30-32]. On the other hand, a respiratory sample that is negative for *Aspergillu*s does not exclude the diagnosis of IPA, since negative cultures have been noted in up to 70% of patients with confirmed IPA [33]. Blood cultures rarely have positive results [34].

MOLECULAR TESTS

Antibody and antigen detection

In theory, the detection of antibodies to *Aspergillus* sp. may be a useful aid in the diagnosis of IPA. However, in practice antibody tests are often negative because of the poor immune status of the patients. The detection of the *Aspergillus* cell wall component galactomannan (GM) has raised high hopes. Methods to demonstrate this antigen include a latex agglutination test ("Pastorex Aspergillus", Sanofi Diagnostics Pasteur, Belgium) and a sandwich ELISA ("Platelia Aspergillus", Bio-Rad, Naraes-La-Coquette, France). In comparative studies, the latter of these tests has shown to have the higher

sensitivity [35,36]. Several large prospective studies have shown promising results using the ELISA in serum for the diagnosis of IPA, with sensitivities of around 90% and specificities of 94-98% [37-39]. However, one large, recent study reported a much lower sensitivity (32%), with a specificity of 95% using the test in serum [40]. So, the value of the test for use in serum is not yet clear.
GM detection can also be used in other clinical samples, such as BAL-fluid. The sensitivity of the test in BAL-fluid has been reported to be about 60-85% [41,42]. These studies suggest that the test will represent an important adjuvant in the diagnosis of IPA. Other antigen or metabolite detection tests are currently not clinically applicable [43]. A possible exemption in the future may be the detection of the fungal cell wall component β(1,3)-D-glucan, a test which has been studied especially in Japan as the G-test [44].

Polymerase chain reaction
PCR methods for the detection of *Aspergillus* DNA are currently being developed and evaluated. Positive results have been reported in urine, BAL fluid, serum and whole blood of patients suspected of having IPA [38,45,46]. Especially the use of the test in whole blood has shown promising results, with reported sensitivity and specificity of 89% and 98% respectively [38]. Unfortunately, there is currently no well-standardised technique that can be routinely applied appropriately in most clinical laboratories. Problems with specificity are an important drawback to the widespread use of these very sensitive tests, since fungal spores are ubiquitous present in the environment, including PCR reactants [47].

TREATMENT

The mortality rate of IPA remains high despite recent advances in therapy. Antifungal treatment should be started as soon as there is clinical suspicion of IPA, since early therapy improves outcome [48]. However, the outcome of treatment not only depends on early diagnosis and start of treatment, but also on the absence of dissemination and the recovery of the underlying host defence defect, such as the resolution of neutropenia or the tapering of immunosuppressive therapy [49,50].
Until recently, the most widely used drug for the treatment of IPA has been amphotericin B (AMB). It's usual dose is 0.6 to 1.5 mg/kg. Even with optimal treatment, the response rate is still disappointing and varies between 33% and 54% in leukaemia and bone-marrow transplant patients [6].

Lipid formulations of AMB
AMB-desoxycholate (Fungizone®) causes serious side effects such as nephrotoxicity, electrolyte disturbances, and infusion related reactions. Newer lipid-based preparations of AMB have been introduced in an effort to minimise the side effects of AMB. Lipid

formulations show a slightly reduced activity when compared with regular AMB, together with clinically significant reduction of toxicity, especially nephrotoxicity [51]. However, the daily acquisition prices of lipid formulations of AMB are much higher than that of AMB – desoxycholate. At this moment there are three commercially developed lipid formulations, all with different confirmations and pharmacokinetics [52]. AMB- lipid complex (ABLC, Abelcet®) consists of tightly packed ribbon-like structures of bilayered membranes containing AMB. AMB-colloidal dispersion (ABCD, Amphocil ® / Amfotec®) is composed of disk-like structures of cholesteryl sulphate complexed with AMB. Liposomal AMB (L-AMB, AmBisome®), the only true liposomal product, consists of small uniformly sized unilamellar vesicles with a diameter of 80 nm.

Of these three lipid compounds L-AMB seems to be the most promising since it is the only lipid compound of that has shown to have clinical response rates superior to that of AMB [53]. To date, the optimal dose of L-AMB in the treatment of IPA is still unclear. Clinical evidence indicates that AMB (both AMB deoxycholate and lipid formulations) may produce a dose response in IPA cases [54,55]. This concept was challenged in a randomised trial which compared L-AMB given at 1 mg/kg/day with L-AMB given at 4 mg/kg/day; this study found no difference in the overall response rate [56]. However, in the subset of patients with documented IPA, the response rate was 37% and 58% in the 1 and 4 mg/kg/day groups, respectively. Therefore, the optimal dosing regime of L-AMB remains an interesting subject of investigation.

Other antifungals

Azoles

Currently, the most important azoles that are used for the treatment of IPA are itraconazole and voriconazole. Itraconazole's fungicidal activity is not as efficient as that of AMB, because inhibition of sterol synthesis takes longer than directly creating channels in the cell membrane [57]. To overcome problems with variable absorption, itraconazole has now been solubilised in cyclodextrin, with substantial improvement as an oral solution [58,59]. Also, a new i.v. formulation was recently approved by the US FDA for the treatment of IPA in patients who are intolerant or have infections refractory to AMB.

Voriconazole is a new second-generation triazole synthetic derivative of fluconazole. Both fungicidal and fungistatic activity against *Aspergillus* have been demonstrated [60]. Voriconazole shows +/- 90% bioavailability, and has good CSF penetration [61]. The main side effects of voriconazole include reversible visual disturbances in as many as 33% of treated patients, occasional elevated hepatic transaminases and occasional skin reactions likely due to photosensitization [60,62].

The newest antifungal agents in the azole group include posaconazole and ravuconazole. The clinical value of these agents in the treatment of IPA is still in an early stage of investigation.

Echinocandins

Caspofungin is a water-soluble semisynthetic derivative of the natural product pneumocandin B [63]. The drug blocks the synthesis of β(1,3)-D-glucan, which is an essential component of the cell wall of numerous fungal species. It has linear pharmacokinetics, is hepatically excreted, and has few side effects. Parenteral administration must be used because of the low bioavailability when administered orally [64]. Fungal growth is required for killing to occur, and the rate of killing is significantly longer than for AMB [65]. Several animal models show equivalent efficacy of caspofungin and AMB for treatment of IA [66]. Caspofungin was approved by the US FDA in February 2001 and is indicated for patients with refractory aspergillosis or intolerance to other therapies.

The newest echinocandins are micafungin and anidulafungin, which are still in primary stages of investigation [67].

ANIMAL MODEL OF IPA

Clinical studies on the diagnosis and treatment of IPA are difficult and time- and money consuming. First, large numbers of high-risk patients in a study are needed to obtain sufficient power, because only a minority of these high-risk patients develops the disease. Second, it is hard to identify patients with proven IPA, because diagnosis is difficult and proven cases are rare. Finally, the appearance of the disease may be different in different types of patients. Consequently, animal models of IPA may be helpful in the investigations to improve diagnosis and management of this disease.

A number of animal models of IA have been described. A considerable proportion of these models are mice models of systemic IA, in which *Aspergillus* conidia are administered by the intavenous route [68]. However, one could argue that the natural route of infection in patients is through the respiratory tract, and these models may therefore lack clinical correlates. Also, in many models immunosuppression is achieved by corticoids. This may make extrapolation of data to neutropenic patients questionable, especially in studies on pathogenesis. Only a few animal models combine neutropenia by chemotherapy with inoculation through the respiratory route [69-72]. In our laboratory, an animal model was developed in rats by Leenders et al., which also combines these features [73,74]. The model was based on the rat model of *Klebsiella pneumoniae* pneumonia, described by Bakker-Woudenberg et al. [75]. Granulocytopenia in the model of IPA was induced by repeated injections of 90 mg/kg cyclophosphamide intraperitoneally (i.p.) at 5 days before and 60 mg/kg at 1 day before and 3, 7 and 11 days after fungal inoculation. This resulted in a persistent neutropenia

(granulocytes < 0.1 x 10^9 / L).. At day 0, animals were inoculated with 10^4 conidia *A. fumigatus* in the left lung. This resulted in a left-sided IPA with 100% mortality around day 9 after fungal inoculation. Fungal dissemination to the contra-lateral right lung and other organs occurred from around day 5 after fungal inoculation. Treatment in the model with a standard dose of 1 mg/kg AMB daily (started at 40 h after fungal inoculation) resulted in 10-20% survival at day 11 after inoculation. This animal model closely mimics the clinical situation in humans in various aspects. First, in humans pulmonary aspergillosis is often initially detected in one of both lungs and then progresses to the contra-lateral lung. Second, the chemotherapy-induced granulocytopenia of < 0.1 x 10^9/L is very similar to the situation in the most important patient group at risk for IPA, i.e. haematological patients receiving chemotherapy. Third, standard treatment with AMB in the model still results in relatively high mortality, which is comparable to the situation in persistently neutropenic patients with IPA. Finally, treatment in the model is started when hyphal growth of the fungus is present, and the infection is already established.
In experiments preceding the work described in the following chapters, the animal model was slightly modified for further optimising. The first dose of cyclophosphamide at 5 days before fungal inoculation was lowered to 75 mg/kg. Further, antifungal treatment was started earlier, at 16 h after fungal inoculation (fungal growth was present at this time point). These changes resulted in 100% mortality of untreated rats around day 11 after inoculation and around 50 % mortality of AMB-treated rats at day 11. This modified model allowed us to better compare other treatment regimes, either superior or inferior, to standard treatment with AMB.

OUTLINE OF THE THESIS

To improve outcome in patients with IPA, more insight into the pathophysiology, diagnosis, treatment and monitoring of IPA is warranted. In this thesis, we address these issues using both the animal model and patients.

The aim of this thesis is to answer the following questions:

- What are the changes in general physiology and lung mechanics caused by the disease, and what causes death in the rat model?
- What parameters of fungal infection are most useful to describe the course of the disease and to monitor antifungal treatment?
- What is the function of the immune system, especially cytokines, in IPA and what is the effect of antifungal treatment on the expression of cytokines?
- What is the value of PCR, galactomannan detection and scintigraphic imaging for an early diagnosis of IPA?
- How can efficacy of antifungal treatment be optimised by using AMB and lipid formulations of AMB?

In **Chapter 2**, clinical symptoms and general and pulmonary pathophysiology of the disease are investigated in the rat model. **Chapter 3** describes the kinetics of several parameters of fungal infection and cytokines in the model, and the effect of antifungal treatment on these parameters. In **Chapter 4**, the value of PCR and galactomannan detection for the diagnosis of IPA are compared in the animal model. **Chapter 5** is a clinical study, investigating the value of galactomannan for diagnosing IPA in neutropenic patients. In **Chapter 6**, several scintigraphic techniques are compared for detecting IPA in the rat model, whereas the most useful of these techniques is further evaluated in the model in **Chapter 7.** In **Chapter 8**, treatment regimes using L-AMB, monotherapy or in combination with AMB, are compared for their efficacy in treating IPA in the rat model.

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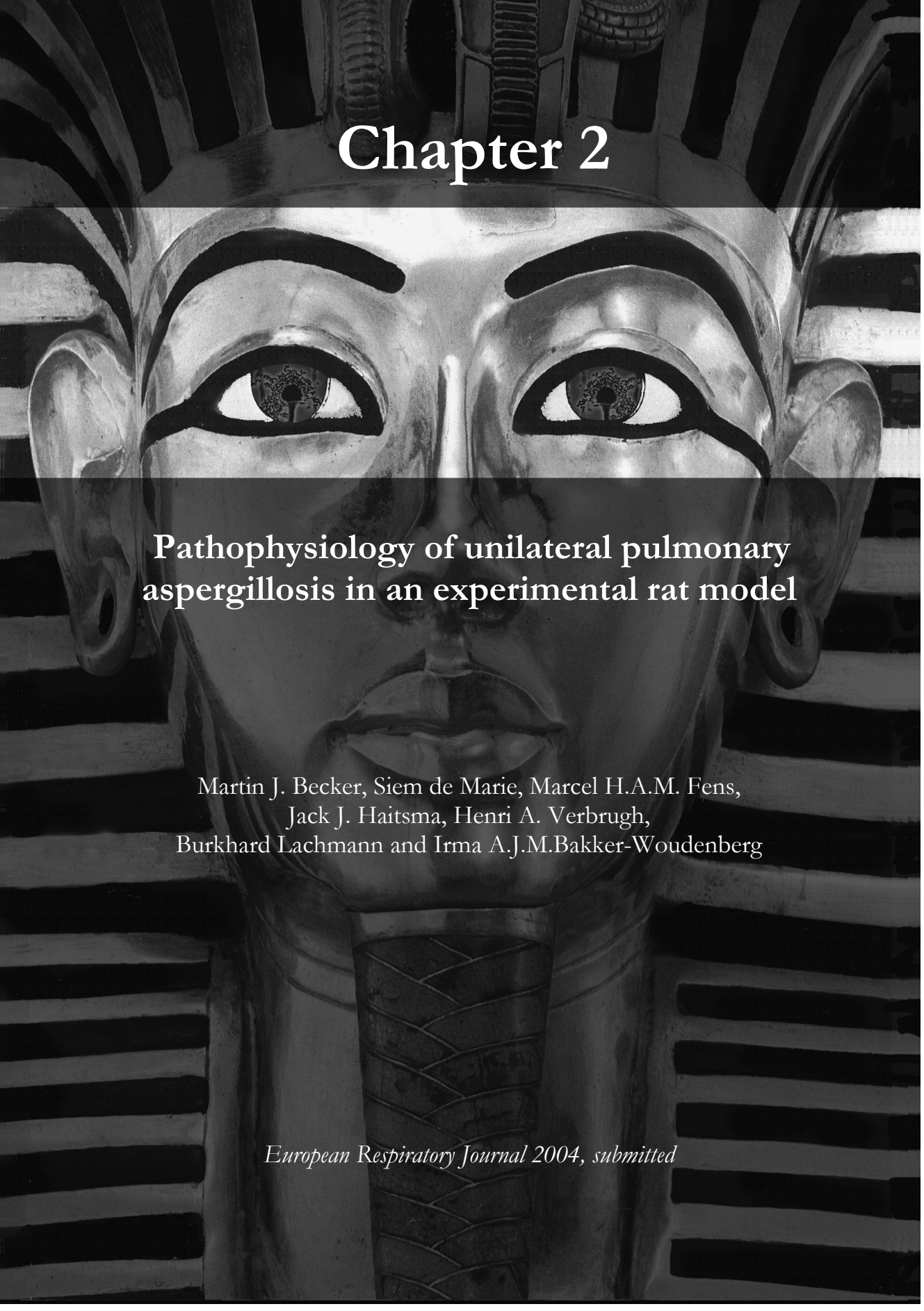

Chapter 2

Pathophysiology of unilateral pulmonary aspergillosis in an experimental rat model

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ABSTRACT

Question of the study: Because little is known about the pathophysiology of invasive pulmonary aspergillosis (IPA), we examined changes in general physiology and lung mechanics during this disease in an animal model.

Materials and methods: In a model of fatal left-sided IPA, 19 persistently neutropenic rats were monitored for clinical signs including body temperature, body weight and respiratory distress. A separate group of 9 rats with IPA was used for measurements of arterial blood pressure, arterial O_2 and CO_2 pressure, lung compliance and surfactant function.

Results: Body temperature and body weight decreased, whereas respiratory distress increased during progression of the disease. Compared to uninfected controls, in rats with IPA arterial blood pressure and lung compliance were significantly lower, and left lung minimal surface tension was significantly higher. Right lung surfactant function was not affected. Arterial O_2 and CO_2 pressures were not different between rats with IPA and uninfected controls.

Answer to the question: Infection with *A. fumigatus* in neutropenic rats resulted in hypothermia, body weight loss and respiratory distress. Loss of left lung function was probably compensated by the uninfected right lung, even in a late stage of the disease. Circulatory failure was a major feature in the terminal phase of the infection.

INTRODUCTION

An increase in the number of immunocompromised hosts in the last decades has led to an increase in the number of severe fungal infections such as invasive pulmonary aspergillosis (IPA) [1]. The most common and best-characterised risk factor for IPA is neutropenia. The mortality rate of IPA in neutropenic patients remains high and exceeds 50% despite antifungal treatment [2]. Therefore, there is a need to expand our understanding of pathogenesis and pathophysiology of IPA during neutropenia in order to develop better strategies of intervention.
Histopathologically, IPA is a narcotising pneumonitis characterised by hyphal proliferation in the pulmonary parenchyma and invasion of pulmonary bronchi and blood vessels, resulting in thrombosis and hemorrhagic infarction. Haematogenous extrapulmonary dissemination occurs especially to the brain, liver and kidneys [3]. However, little is known about the way in which this fungal disease influences pulmonary function and general physiology. Studies in patients with IPA are hampered by difficulties in identifying patients with proven IPA and by the impact of co-morbidity in these patients. Animal models of IPA do not have these ancillary problems and may therefore be helpful in studying the pathophysiology of this disease.
In our laboratory, we have developed a model of unilateral IPA in rats that closely mimics human disease. The model is characterised by prolonged severe granulocytopenia, inoculation through the respiratory route, fungal broncho- and angio-invasion and dissemination of the fungus from the lung to other organs [4-7]. In this model we examined the changes in general physiology and lung mechanics caused by the disease.

MATERIALS AND METHODS

The experimental protocols adhered to the rules laid down in The Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The present protocols were approved by the Institutional Animal Care and Use Committee of the Erasmus Medical Center Rotterdam.

Infection model of IPA

Our animal model, first described by Leenders et al. [4], was used with a few modifications. Specified pathogen- free female RP strain albino rats (18-25 weeks old, 185-225 g) were employed. Profound granulocytopenia was induced by intraperitoneal (i.p.) administration of 75 mg/kg cyclophosphamide (Sigma-Aldrich Chemie, Steinheim, Germany) 5 days before fungal inoculation, followed by repeated doses of 60 mg/kg 1 day before and 3 and 7 days after inoculation. This protocol resulted in granulocyte counts of less than 0.1×10^9 / L on the day of fungal inoculation and

thereafter. To prevent bacterial superinfections, animals were given daily doses of 40 mg/kg amoxicillin intramuscularly (i.m.) starting 1 day before inoculation and a 6 mg/kg dose of gentamicin i.m. on the day of inoculation. In addition, rats received ciprofloxacin (660 mg/L) and polymyxin B (100 mg/L) in their drinking water throughout the experiment. The rats were anaesthetised with fentanyl citrate (Hypnorm, Janssen, Belgium), 0.0315 mg / rat i.m. together with sodium pentobarbital (Nembutal, Sanofi Sante, the Netherlands), 4.5 mg / rat i.p. The left main bronchus was intubated. A cannula was passed through the tube and the left lung was inoculated with 6×10^4 *Aspergillus fumigatus* conidia in 0.02 ml phosphate buffered saline (PBS, pH 7.4). This resulted in a left-sided IPA. A clinical isolate of *A. fumigatus* was used that was originally isolated from an immunocompromised patient with IPA. Survival of rats was monitored twice daily.

The experimental protocols used in this study adhered to the rules laid down in The Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The present protocols were approved by the Institutional Animal Care and Use Committee of the Erasmus Medical Center Rotterdam.

Clinical parameters

Clinical parameters were measured as described before [8].

Body temperature was measured using the ELAMS® - system (Electronic Laboratory Animal Monitoring System, BioMedic Data Systems, Inc. Seaford DE, USA). This system consisted of a portable data acquisition system connected to a detectable scanner wand (DAS-5002), and implantable programmable temperature transponders (IPTT-100).

Body weight was measured daily throughout the experiment.

Respiratory distress was scaled into three categories. Normal breathing was defined as a normal respiratory rate (85-110 / min) and no visible respiratory distress. Moderate respiratory distress was seen as impaired ability to expand the thorax without reduction of respiratory frequency. Severe respiratory distress was seen as a strongly impaired ability to expand the thorax ("gasping") with reduced respiratory frequency (30-75/min).

Wheezing was defined as an audible breathing sound, mostly a squeaking sound. This parameter was scored as either present or absent.

Macroscopic pulmonary lesion size

In order to measure the size of the haemorrhagic lesion of the affected lung, photographs were taken immediately after dissection. The photograph was taken from the ventral position of the lung. The morphologic extension of the lesion was measured on the photographs and expressed as a percentage of the total lung surface.

Measurements of blood pressure and pulmonary function

At day 7 after fungal inoculation, rats were anaesthetised with 0.0315 mg fentanyl citrate, 1 mg fluanisone and 22.2 mg/kg natrium pentobarbital A catheter was inserted into the left carotid artery and after tracheotomy, a tube was inserted into the trachea. Subsequently, rats were allowed to breathe freely until a regular breathing pattern was established. Mean arterial blood pressure was measured using a Statham P23XL transducer (Spectramed, Oxford, CA, USA) and recorded (Siemens Sirecast 404-1, Danvers, MA, USA). In blood samples obtained from the carotid artery, blood O_2 tensions were measured using an ABL 505 Radiometer (Copenhagen, Denmark).

After the animals were killed, lung weight was determined and a static pressure-volume curve for both lungs was recorded using conventional techniques [9]. Maximal compliance (C_{max}) was defined as the steepest part of the pressure-volume curve [10].

Broncho- alveolar lavage (BAL) was performed by separate lavage of the infected left and uninfected right lung with 5 ml saline-$CaCl_2$ 1.5 mmol /L. Lavage fluid was subsequently centrifuged to remove cells and cellular debris.

Minimal surface tension measurements

Minimal surface tensions of BAL samples containing surfactant were measured using a modified Wilhelmy balance (E. Biegler GmbH, Mauerbach, Austria) which keeps the temperature constant at 37 °C [11]. The trough was filled with warm saline (37 °C) and calibrated. After calibration, 500 µL of the lavage sample was placed on the surface. Minimal surface tension was measured after 3 cycles at 20% surface area, and expressed as milli Newton/meter (mN/m).

RESULTS

Survival and clinical signs

One group of persistently neutropenic rats with unilateral IPA (n=19), one control group of neutropenic rats without infection (n=5) and one control group of naive rats (n=5) were used to monitor survival and clinical signs. Infection with *A. fumigatus* resulted in 42 % mortality at day 7 after fungal inoculation and 100% at day 11 (Figure 1). Neutropenic, uninfected rats and naive rats all survived.

The average body temperature of the rats on day 0 was 36.9 °C. In neutropenic rats with IPA, a decrease in body temperature was observed during the course of the disease, with average temperatures declining to 30.1 °C on day 9 after inoculation (Figure 2). All rats in which body temperature dropped below 34 °C died within 24 h after that measurement. Body temperature in neutropenic, uninfected rats and naive rats remained around 37 °C throughout the experiment.

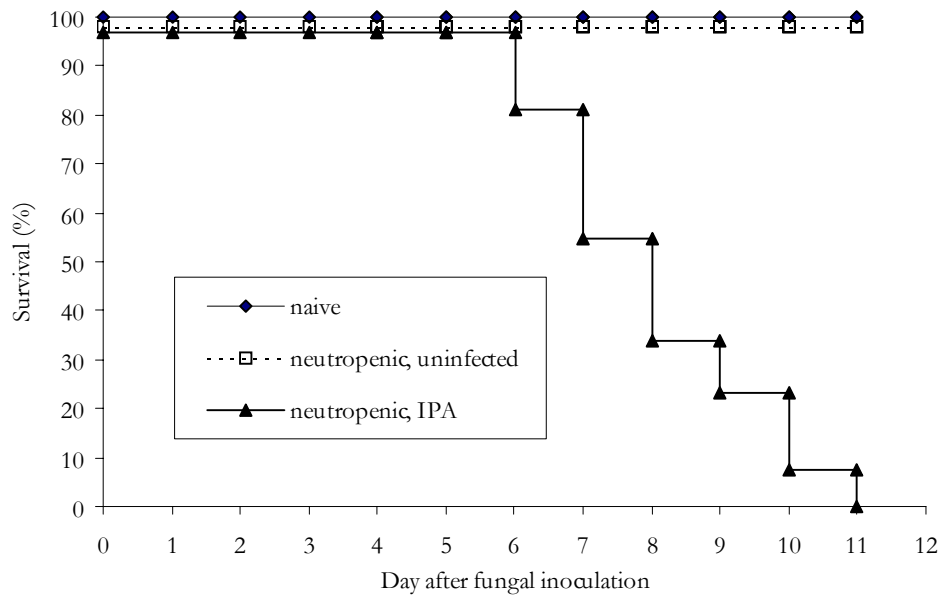

Figure 1. Survival of neutropenic rats with IPA (n = 19), neutropenic uninfected rats (n = 5) and naive rats (n = 5).

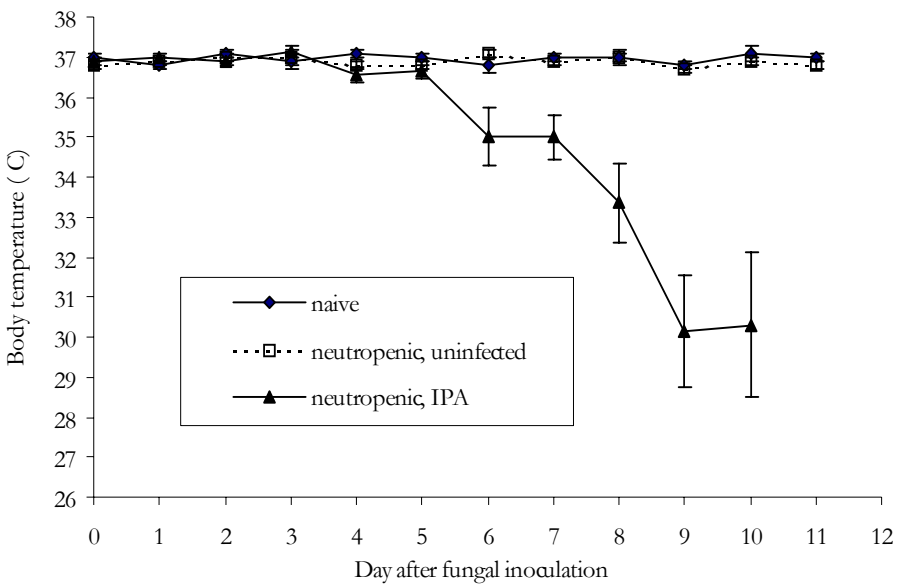

Figure 2. Body temperature of neutropenic rats with IPA (n = 19), neutropenic uninfected rats (n = 5) and naive rats (n = 5).

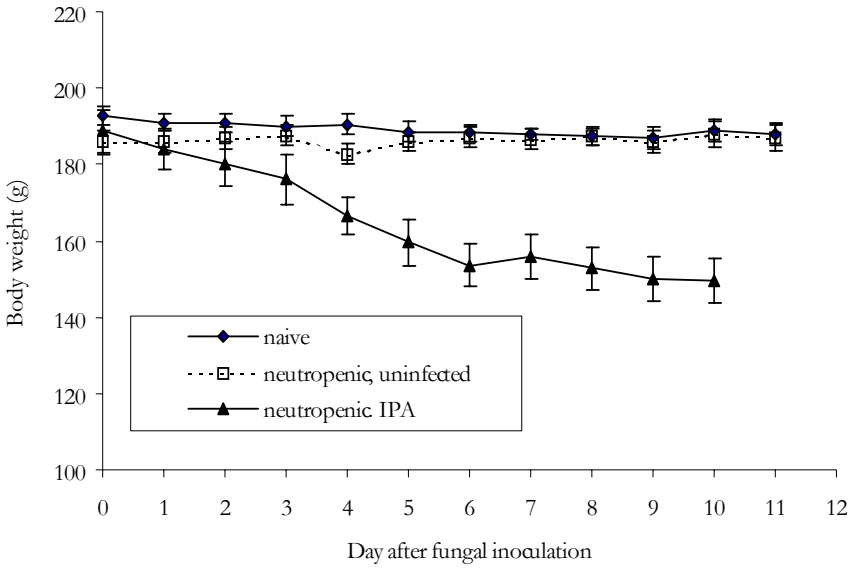

Figure 3. Body weight of neutropenic rats with IPA (n = 19), neutropenic uninfected rats (n = 5) and naive rats (n = 5).

The average body weight of naive rats remained about constant over time (Figure 3). The initial body weight of neutropenic, uninfected rats was slightly lower than that of

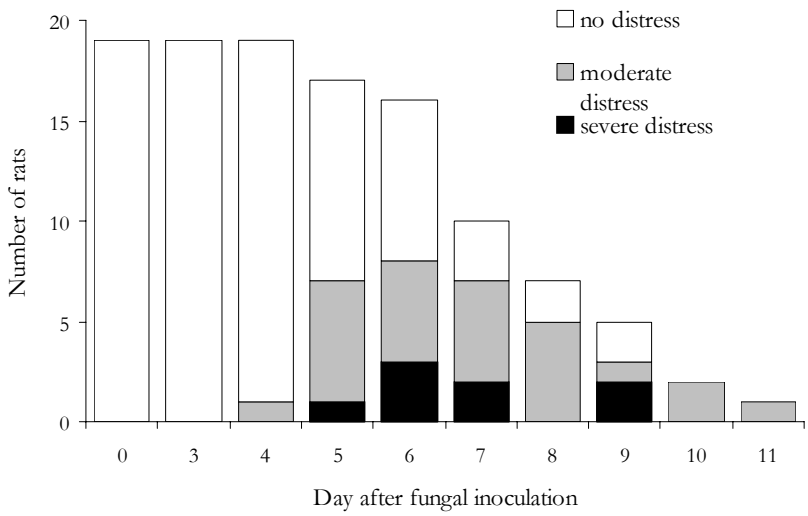

Figure 4. Respiratory distress in neutropenic rats with IPA (n = 19).

naive rats at day 0, but no decrease in body weight was seen thereafter. In contrast, in neutropenic rats with IPA, a strong decrease in body weight occurred, up to 21% of the initial body weight at day 10 after fungal inoculation.
In naive and neutropenic, uninfected rats, the respiratory rate was around 100/min throughout the experiment (data not shown). Starting in a proportion of rats at day 4 after fungal inoculation, animals showed moderate or severe respiratory distress. The proportion of rats with respiratory distress increased with progression of the disease (Figure 4). Severe respiratory distress had a high positive predicting value (80%) for death within 24 h. However, 4 rats (21%) died without signs of respiratory distress at any time during the disease process. Wheezing was observed in a minority of rats (Figure 5). The predictive value of wheezing for death within 24 h was 100%.

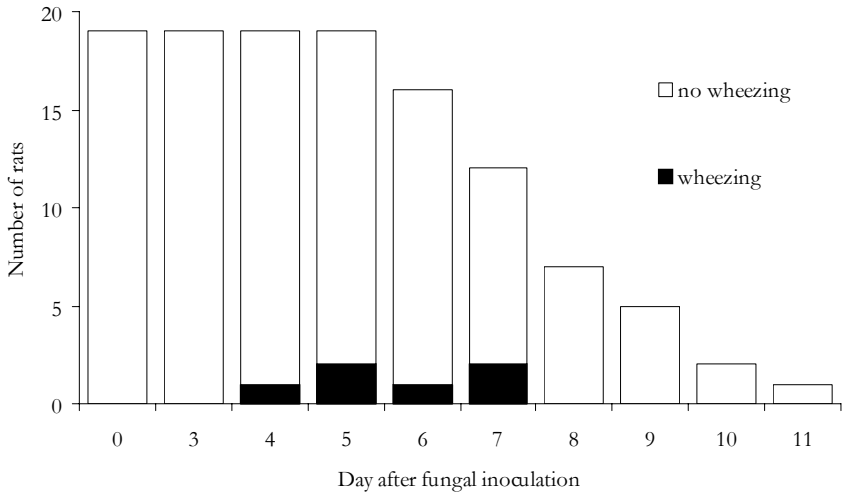

Figure 5. Wheezing in neutropenic rats with IPA (n = 19).

Pulmonary function and blood pressure
One group of neutropenic rats with IPA (n=9), one control group of neutropenic rats without infection (n=7) and one control group of naive rats (n=5) were used to measure pulmonary function and blood pressure. Measurements were performed at day 7 after inoculation (Table 1). At this time point, body temperature was significantly lower in neutropenic rats with IPA compared to both control groups ($P < 0.02$ for both comparisons). All neutropenic rats with IPA showed signs of moderate or severe respiratory distress at day 7 (Figure 4). Despite this respiratory distress, the arterial O_2 pressure was not decreased in the infected rats. In addition, the arterial CO_2 pressure showed no difference between the three groups.

Systolic, diastolic and mean arterial pressures were much lower in neutropenic rats with IPA compared to the control groups ($P < 0.003$ for both comparisons). On average, the size of the macroscopic pulmonary lesion of the left lung in neutropenic rats with IPA was 67% of the total lung surface. Neutropenic, uninfected rats and naive rats had no pulmonary lesions. Lung compliance, measured over left and right lungs simultaneously, was significant lower in neutropenic rats with IPA ($P<0.001$ for both comparisons). The minimal surface tension of BAL- fluid samples from the infected left lungs of neutropenic rats with IPA was higher than that of uninfected neutropenic rats ($P=0.01$). The weight of the infected left lung in neutropenic rats with IPA was increased compared to both control groups ($P<0.002$ for both comparisons). In contrast, the weight of the uninfected right lung was similar over the three groups.

Table 1. Pulmonary functions and blood pressure in neutropenic rats with IPA at day 7 after inoculation, neutropenic uninfected rats and naive rats

	Neutropenic rats with IPA	Neutropenic uninfected rats	Naive rats
No of rats	9	7	5
Systolic arterial blood pressure (mm Hg)	71.2 ± 5.3*	105.4 ± 3.1	125.8 ± 7.1
Diastolic arterial blood pressure (mm Hg)	57.0 ± 5.3*	87.0 ± 1.9	104.4 ± 5.9
Mean arterial blood pressure (mm Hg)	62.6 ± 5.6*	117.0 ± 2.3	125.8 ± 7.1
PaO_2 (mm Hg)	96.3 ± 9.6	87.5 ± 7.7	91.5 ± 6.2
$PaCO_2$ (mm Hg)	49.2 ± 5.1	51.2 ± 5.2	50.7 ± 4.9
Weight infected left lung (g)	1.40 ± 0.14*	0.68 ± 0.06	0.70 ± 0.05
Weight uninfected right lung (g)	1.28 ± 0.09	1.26 ± 0.12	1.14 ± 0.08
Macroscopic pulmonary lesion size (%)	67 ± 9	0 ± 0	0 ± 0
C_{max} (ml / cm H_2O * kg)	3.57 ± 0.23*	6.67 ± 0.2	6.39 ± 0.24
Minimal surface tension (mN / m) infected left lung	30.9 ± 2.0■	19.7 ± 2.7	n.d
Minimal surface tension (mN / m) uninfected right lung	18.1 ± 1.4	18.7 ± 2.9	n.d.

Data are presented as mean ± SEM

* significant difference ($P< 0.05$) compared to neutropenic, uninfected rats and compared to naive rats

■ significant difference ($P < 0.05$) compared to neutropenic, uninfected rats

DISCUSSION

Few data are available on the pathophysiology of IPA in humans or animals with neutropenia. The present study describes the general and pulmonary pathophysiology of IPA in an experimental rat model of unilateral IPA. In earlier studies in this model, it was found that infection with *A. fumigatus* resulted in fungal tissue-, broncho- and angio -invasion causing increasing hemorrhagic infarcts and tissue necrosis over time [5,7].
In the current study a 100% mortality of neutropenic rats with IPA was seen. This is comparable to neutropenic patients with IPA, in which mortality approaches 100% when the disease is left untreated [2].
In contrast to fever, which is seen in patients with IPA, a decrease in body temperature was observed in the rats. This hypothermia is a phenomenon that is seen in several other infection models in small experimental animals, including models of fungal infection [12-14]. Because of the high body surface-body mass ratio of small animals, a relatively high heat production is necessary to maintain a high and steady temperature, and therefore much energy would be needed for the sustenance of fever. The multiplication and pathogenicity of many infectious agents is also suppressed at low and not only at high temperature. Therefore, it has been suggested that in small animals cryexia may be an alternative to fever for coping with infections [15].
Neutropenic rats with IPA experienced a significant loss of body weight during the course of the disease. This weight reduction was at least partially caused by a reduction in food and water intake (unpublished data). In addition, an enhanced catabolic state may have resulted in weight loss, as seen in other rat infection models [16-19].
In the terminal phase of the disease, at day 7 after fungal inoculation, we investigated the lung function, blood pressure and lung pathology in our rat model. Inoculation of *A. fumigatus* into the left lung resulted in pulmonary hemorrhagic lesions that comprised more than 50% of the lung in most animals. This had a dramatic effect on lung mechanics as demonstrated by a decrease in lung compliance. Surfactant function was impaired in the infected left lung, demonstrated by a significant increase in minimal surface tension in BAL-fluid, resulting in loss of alveolar stability and finally atelectasis formation. The impaired surfactant function may be caused by a direct influence of *A. fumigatus* hyphae on surfactant, since the fungus is able to bind surfactant proteins *in vitro* [20]. However, the accumulation of plasma proteins in the alveoli, dose dependently impairs the endogenous surfactant system [9]. In the left lung of the rats in our study a large pulmonary hemorrhagic lesion of more than 50% was present, strongly suggesting accumulation of these plasma proteins.
There have been conflicting reports on contralateral lung damage by unilateral pulmonary infection by induction of cytokines and/or neutrophil influx.
Dehoux et al. demonstrated that compartmentalisation is preserved during unilateral pneumonia [21], whereas others found that unilateral pulmonary infections could cause contralateral lung damage by induction of cytokines and neutrophil influx [22].

In our model, lung damage was limited to the infected lung demonstrated by both the normal minimal surface tension and lung weight in the uninfected right lung which were not increased in neutropenic rats with IPA compared to that in lungs of neutropenic, uninfected rats and naive rats. This suggests that the right lung was not affected during the disease in neutropenic rats with IPA.
The deteriorated lung mechanics and surfactant impairment in the infected lungs of rats with IPA probably caused the change in respiratory rate that was observed in most rats. In patients with IPA respiratory distress can occur, especially in patients that succumb to the infection [23]. However, the arterial oxygen pressure in our model was not decreased in neutropenic infected rats in the terminal phase of the disease compared to neutropenic, uninfected rats and naive rats. This suggests that the uninfected right lung in neutropenic, infected rats with IPA compensates the loss of function of the left lung, at least until a late stage in the disease. This is probably accomplished by hypoxic pulmonary vasoconstriction resulting in no-shunt flow through the diseased left lung and only flow through the aerated right lung tissue, thus preserving oxygenation [24]. In all animals arterial CO_2 was slightly increased caused by the respiratory depression due to anaesthesia.
Bacterial pathogens can cause septic shock in man, which is a distributive shock with moderate-to- high cardiac output in the presence of a low systemic vascular resistance [25]. The cytokine TNF-α is thought to play an important role in the circulatory dysfunction, increased microvascular permeability and metabolic derangements [26,27]. Few data exist on circulatory dysfunction and the development of shock in patients with IPA. However, some case reports mention the development of circulatory failure in these patients [28,29]. The neutropenic rats with IPA in our model developed a circulatory failure, as demonstrated by a marked decrease in arterial blood pressure on day 7 after fungal inoculation. The mechanism of development of circulatory failure in fungal infections may be different from bacterial infections. Shock in rats with candidemia was found to be TNF-α independent [12], and earlier investigations in our rat model of IPA did not reveal increases of TNF-α in lung or serum [30]. Thus, circulatory failure in IPA may be induced by circulating fungal virulence factors or host mediators other than TNF-α. Future studies should further explore the role of circulatory failure in IPA.
In conclusion, unilateral pulmonary infection with *A. fumigatus* in neutropenic rats resulted in hypothermia, loss of body weight and respiratory distress. Infection caused changed lung mechanics and surfactant dysfunction of the infected lung but loss of the left lung function was probably compensated by the uninfected right lung, even in a late stage of the disease. Animals probably died from circulatory failure and not from direct pulmonary complications, although severe unilateral lung pathology was present.

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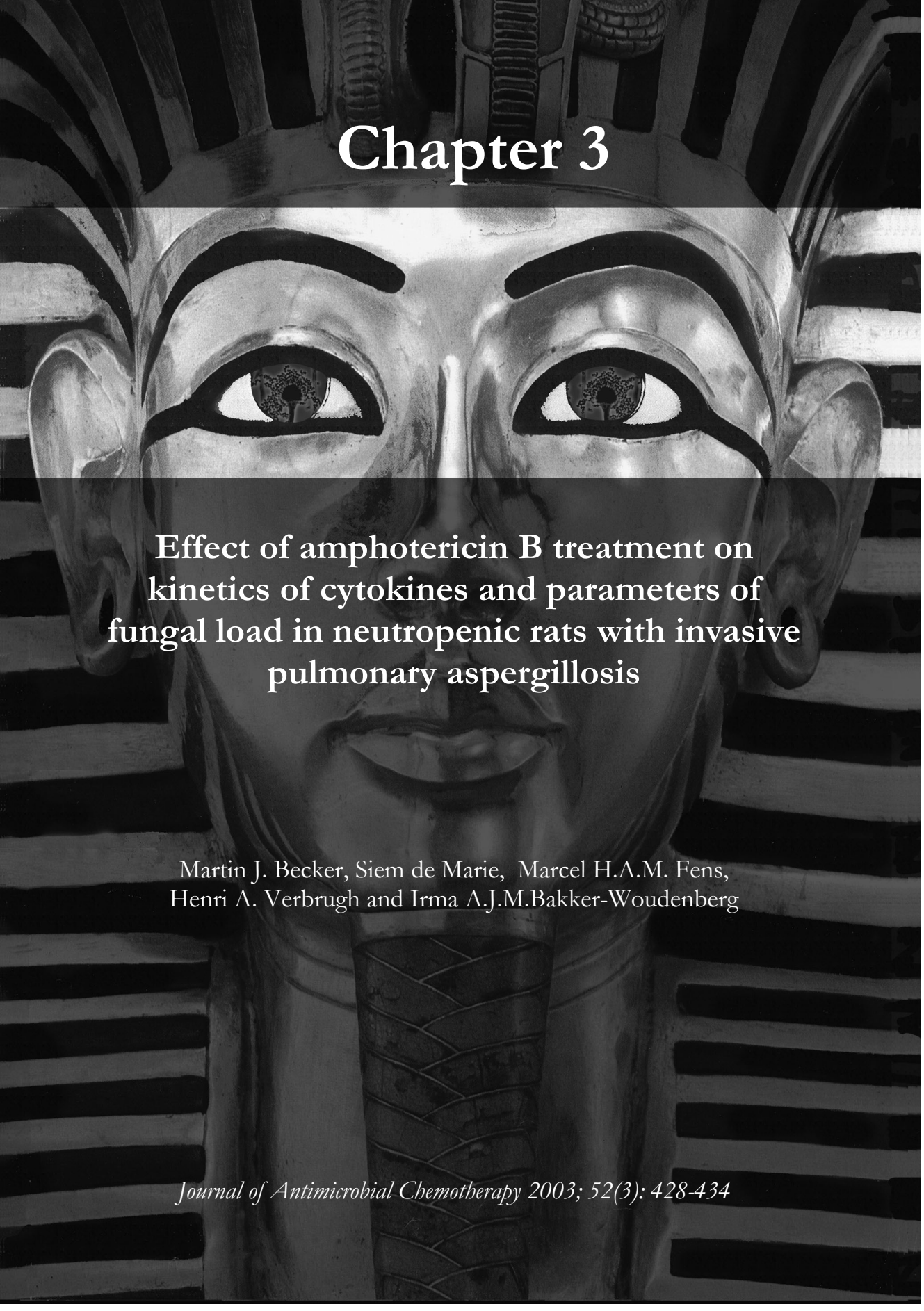

Chapter 3

Effect of amphotericin B treatment on kinetics of cytokines and parameters of fungal load in neutropenic rats with invasive pulmonary aspergillosis

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ABSTRACT

Objective: To acquire insight into the pathogenesis of invasive pulmonary aspergillosis (IPA) during antifungal treatment with amphotericin B, kinetics of various parameters of fungal load and cytokines were investigated.

Methods: Neutropenic rats with left-sided IPA received either treatment with amphotericin B or remained untreated. At 0, 4, 8, 16, 24, 48, 72 and 120 h after fungal inoculation rats were dissected. Size of macroscopic pulmonary lesions, numbers of cfu and chitin amounts were determined in the infected left lung. Galactomannan concentrations were measured both in the left lung and serum. The cytokines tumor necrosis factor (TNF)-α, interleukin (IL) 1-β, IL-6, interferon (IFN)-γ, IL-4 and IL-10 and the chemokines macrophage inflammatory protein (MIP)-2 and monocyte chemoattractant protein (MCP)-1 were quantitatively determined by ELISA in the infected left lung, uninfected right lung and serum.

Results: Amphotericin B treatment of IPA resulted in changed aspect of pulmonary lesions and significantly reduced levels of left lung chitin (72 and 120 h), left lung galactomannan (72 and 120 h) and serum galactomannan (120 h), but not left lung cfu, compared with untreated, infected rats. In addition, amphotericin B treatment resulted in a significant decrease in levels of left lung IL-6 (72 and 120 h), MIP-2 (120 h) and MCP-1 (120 h). No local or systemic increases of TNF-α, IL1-β or IFN-γ were observed during infection.

Conclusion: It is concluded that treatment with amphotericin B results in decreased fungal load in the infected lung. Probably, this reduction in fungal load results in a decreased local inflammatory response as measured by decreased levels of IL-6, MIP-2 and MCP-1 in the infected lung.

INTRODUCTION

Aspergillus fumigatus is a ubiquitous fungus that can cause invasive pulmonary aspergillosis (IPA) in immunocompromised patients. Evidence suggests a recent increase in this disease, which has been attributed to increased use of potent immunosuppressive agents as part of therapy for organ transplantation and cancer chemotherapy in patients [1,2]. The commonest and best-characterized risk factor for IPA is persistent neutropenia [3]. In neutropenic patients with IPA, treatment with amphotericin B remains the regimen of first choice. However, this treatment often fails, with reported response rates below 55% in leukaemia- and bone marrow transplantation patientsc [4]. Greater insight into the pathogenesis of IPA during antifungal treatment may help develop more effective treatment regimens in the future.
In the pathogenesis of IPA, both fungal and host factors play a role. In several animal models of IPA, tissue-invasive growth of the fungus was seen with an increase of fungal load over time [5-7]. Antifungal treatment resulted in a reduction in fungal load and tissue damage [8-10]. With respect to host response, cytokines seem to play an important role. Studies showed that resistance to systemic invasive aspergillosis was associated with increased systemic production of Th1 cytokines such as interferon (IFN)-γ. In contrast, production of interleukin (IL)-4 and IL-10 by interstitial $CD4^+$ Th2 cell was associated with disease progression [11-14]. In addition, local levels of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α, IL-1-β and IL-6 were elevated in animal models of IPA, and neutralization of TNF-α resulted in increased mortality [15,16]. Finally, both the local levels of the C-X-C chemokine macrophage inflammatory protein (MIP)-2 and the C-C chemokine monocyte chemoattractant protein (MCP)-1 were elevated in animal models of IPA, and neutralization of these chemokines or their receptors resulted in decreased fungal clearance [17,18]. Few data exist regarding the impact of antifungal treatment on patterns of cytokine release.
In our laboratory, we have developed an inhalation model of unilateral IPA in persistently neutropenic rats [19]. In this model, we investigated the effect of treatment with amphotericin B on the kinetics of cytokines and parameters of fungal load in rats with IPA.

MATERIALS AND METHODS

Infection model of IPA

The rat model of IPA was used as described before [20,21]. In brief, specified pathogen free female RP strain albino rats (18-25 weeks old, 185-225 g) were employed. Persistent neutropenia was induced by intraperitoneal (i.p.) administration of 75 mg/kg cyclophosphamide (Sigma-Aldrich Chemie, Steinheim, Germany) 5 days before fungal inoculation, followed by repeated doses of 60 mg/kg 1 day before and 3 and 7 days after fungal inoculation. This protocol resulted in granulocyte counts of less than

0.1×10^9 / L on the day of fungal inoculation. To prevent bacterial superinfections, animals were given daily doses of 40 mg/kg amoxicillin intramuscularly (i.m.) starting 1 day before inoculation and a 6 mg/kg dose of gentamycin i.m. on the day of inoculation. In addition, rats received ciprofloxacin (660 mg/L) and polymyxin B (100 mg/L) in their drinking water throughout the experiment. Rats were inoculated with a clinical isolate of *A. fumigatus* originally isolated from an immunocompromised patient with IPA. Infection was established by intubation of the left main bronchus under general anaesthesia. A cannula was passed through the tube and the left lung was inoculated with 6×10^4 *A. fumigatus* conidia suspended in 20 μL of phosphate buffered saline (PBS).
The experimental protocols adhered to the rules laid down in The Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The present protocols were approved by the Institutional Animal Care and Use Committee of the Erasmus Medical Center Rotterdam.

Antifungal treatment
Amphotericin B (Fungizone®) was obtained from Bristol-Meyers BV (Woerden, The Netherlands), and was diluted in 5% dextrose. Fungizone® was administered intravenously via the lateral tail vein in a dose of 1 mg/kg/day. Treatment was started at 16 h after fungal inoculation, a time point at which hyphal growth was established. Treatment was continued for 10 days.

Survival rate
A separate experiment was performed to observe death rate in amphotericin B – treated, infected rats and untreated, infected rats (n = 25 to 28 rats per group). The survival rate was monitored twice daily until day 11 after fungal inoculation.

Parameters of fungal load and cytokines

Parameters of fungal load were compared in amphotericin B - treated, infected rats and untreated, infected rats. Cytokines were determined in these groups, and also in uninfected rats. At designated time points (n = 5 to 6 rats per group per time point), rats were anaesthetized with 50 mg/ kg pentobarbital (Ceva Sante Animale, Maassluis, The Netherlands) i.p. The chest cavity was opened aseptically and the right ventricle was punctured to obtain blood. Subsequently, through the same needle the lungs were perfused with 5 mL phosphate buffered saline (PBS). The infected left lung and uninfected right lung were removed, kept on ice and homogenized separately in 10 mL PBS with 1 x complete protease inhibitor (Roche Diagnostics, Mannheim, Germany) using a tissue homogenizer (The Virtis Co. Inc., Gardiner, NY, USA). The homogenates were centrifuged (10 min 4000 x g, 4 ° C) and supernatants were passed through a 0.45 μm pore size filter (Schleicher and Schuell, Dassel, Germany) and stored

at – 20 ºC for cytokine and galactomannan assays. The pellet was resuspended in 10 mL PBS and used for cfu and chitin assay.

Pulmonary macroscopic lesion and histopathology

Separate groups of rats were used for macroscopic observation and histopathology. The left and right lung were fixed with formalin and embedded in paraffin. Every lung was cut at 3 levels ± 1 mm apart. At every level 2 adjacent sections were obtained, of which one was stained with haematoxylin - eosin (H&E) and the other with Grocott's methenamine silver [22].

Pulmonary macroscopic lesions were classified and measured as described before [23]. In brief, angio-invasive lesions, seen as macroscopic dark-red lesions, were histologicaly characterized by extensive hyphal broncho- and angio-invasion and haemorrhagic infarction. Responsive lesions, seen as macroscopic light-red coloured lesions were histologicaly characterized by the presence of relatively short hyphae and little angio- and bronchio-invasion with resulting less haemorrhagic infarction [23]. The pulmonary lesion size was measured from photographs of the anterior of the lungs taken immediately after dissection. The size of the two types of pulmonary lesions was expressed as percentage of the total left lung surface.

Fungal cultures of organs

Cfu in lungs were counted in 1:10 and 1:100 dilutions of left lung homogenate and 1:10 dilutions of right lung homogenate. Dilutions of homogenates were spread onto Sabouraud agar plates. After incubation at 37 °C for 36 h the cfu were counted. The remaining homogenate was used for chitin assay and galactomannan assay.

Chitin assay

The chitin assay was performed as described by Lehmann et al. [24]. In brief, the lung homogenate was centrifuged (1800 x g, 15 min.), resuspended in 4 mL of 3% sodium lauryl sulfate (SDS, Sigma chemical co., St Louis, USA) and heated at 100 °C for 15 min. After cooling, the pellet was washed once with distilled water, resuspended in 3 mL of 120% KOH solution and heated to 130 °C for 1h. Subsequently, 8 mL of ice-cold 75% ethanol was added, tubes were kept at 4 °C for 15 min and 0.3 mL of Celite suspension (Celite 545; Sigma) was added. After centrifugation (1800 x g, 5 min, 4 °C), the pellet was washed with cold ethanol (40%) and cold distilled water successively and suspended in 0.5 mL of $NaNO_2$ (5%) and 0.5 mL of $KHSO_4$ (95%). After centrifugation (1800 x g, 15 min), volumes of the supernatant were mixed with 12.5% $NH_4SO_3NH_2$ followed by MBTH (3-methyl-benzo-2-thiazolone hydrazone HCl monohydrate; Sigma). After heating for 3 min, the supernatants were cooled, $FeCl_3$ $6H_2O$ (0.83%) was added and were allowed to stand for 30 min. The optical density at 650 nm was read in a spectrophotometer. The chitin content was expressed as micrograms of glucosamine per left lung. Final measurements of chitin were corrected for the loss of volume of homogenate.

Galactomannan assay
Concentrations of galactomannan in organs and serum were measured as described before [20,23]. Briefly, 300 μL of each sample of supernatant of left- or right lung-homogenate or serum was used in a sandwich ELISA (Platelia Aspergillus, Sanofi Diagnostics Pasteur, Belgium). Each plate contained a calibration curve derived from rat serum or lung-homogenate samples containing 0, 1, 1.5, 2, 3, 4, 6, 8 and 12 ng/mL galactomannan. The concentration of galactomannan in positive test samples was expressed as amount of nanograms galactomannan per mL serum or lung homogenate.

Cytokine ELISA
Rat TNF-α, IFN-γ, IL-1β, IL-4, IL-6, IL-10, MIP-2 and MCP-1 were quantified in supernatants of left- and right lung-homogenates and serum using ELISA-kits ("Cytoscreen", Biosource International, Camarillo, USA). Each kit contained a calibration curve with different concentrations of the respective cytokines.

Statistical analysis
Differences in rat survival rate were assessed by log rank test. Differences in parameters of fungal infection and cytokines were assessed by student's T test.

RESULTS

Effect of amphotericin B treatment on rat survival rate
The survival rate of neutropenic rats with IPA was compared in amphotericin B-treated, infected rats and untreated, infected rats. Treatment was started at 16 h after fungal inoculation, at which time hyphal growth was established (data not shown). Per group, 25 to 28 rats were used. Untreated, infected rats died from day 5 after fungal inoculation. Death of all rats had occured at day 11 (Figure1). Treatment with amphotericin B resulted in a significant increase in survival rate ($P < 0.0001$), with the first rats dying at day 6 after fungal inoculation, and 52% survival of rats at day 11.

Effect of amphotericin B treatment on parameters of fungal load and cytokines
Parameters of fungal load and levels of cytokines were compared in amphotericin B-treated, infected rats and untreated, infected rats. Cytokines were also determined in uninfected rats. To avoid selection bias, time points were chosen at which all the rats in the three groups were still alive, i.e. at 0, 4, 8, 16, 24, 48, 72 and 120 h after fungal inoculation. In each group, 5 to 6 rats per time point were used.

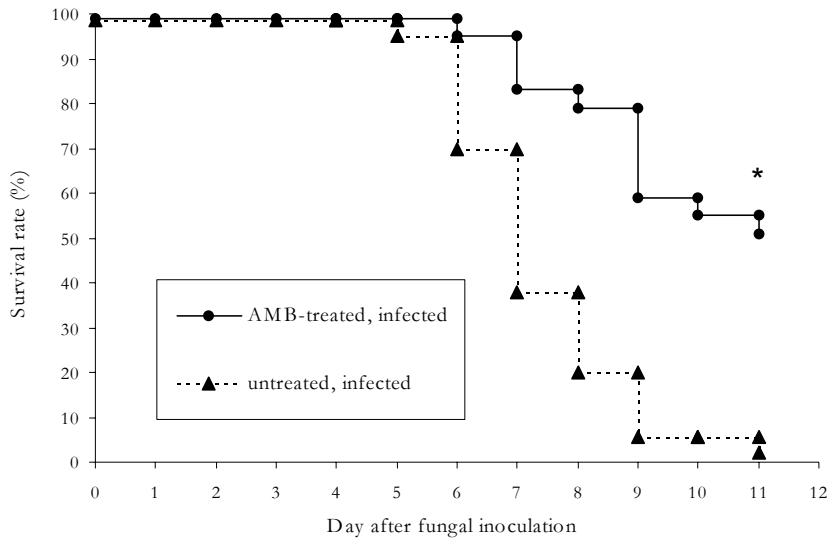


Figure 1. Survival of rats with IPA. Rats received either treatment with AMB 1 mg/kg/day i.v., starting at 16 h after fungal inoculation, or remained untreated. * = significantly different compared to untreated, infected rats ($P<0.05$)

Pulmonary macroscopic lesions and histopathology

In untreated, infected rats, dark-red coloured haemorrhagic pulmonary lesions were seen at 48 h and later, increasing in size over time. At 120 h after fungal inoculation the size of these lesions comprised on average 49 ± 4% of the total left lung surface. Histologicaly, these "angio-invasive " lesions showed extensive fungal broncho- and angio-invasion and tissue haemorrhagia. In amphotericin B- treated, infected rats, pulmonary lesions also increased over time, but the aspect of these lesions was different. A shift was seen from dark-red coloured angio-invasive lesions in untreated, infected animals to "responsive" lesions in amphotericin B-treated, infected rats. Histologically, these responsive lesions showed shorter hyphae, reduced fungal broncho-and angio-invasion and less tissue hemorrhagia compared to untreated, infected rats. At 120 h after fungal inoculation, these responsive lesions comprised 46 ± 15% of the total left lung surface.

Number of cfu

In untreated, infected rats no increase was seen in number of cfu in the infected left lung over time, despite progression of the fungal infection (Figure 2). Treatment with amphotericin B did not significantly reduce number of left lung cfu compared to untreated, infected rats. Right lungs were positive for cfu in two untreated, infected rats at 120 h after fungal inoculation (mean: 1.15 ^{10}log cfu). In amphotericin B -treated,

infected rats all right lungs were negative. The difference was not significant (data not shown).

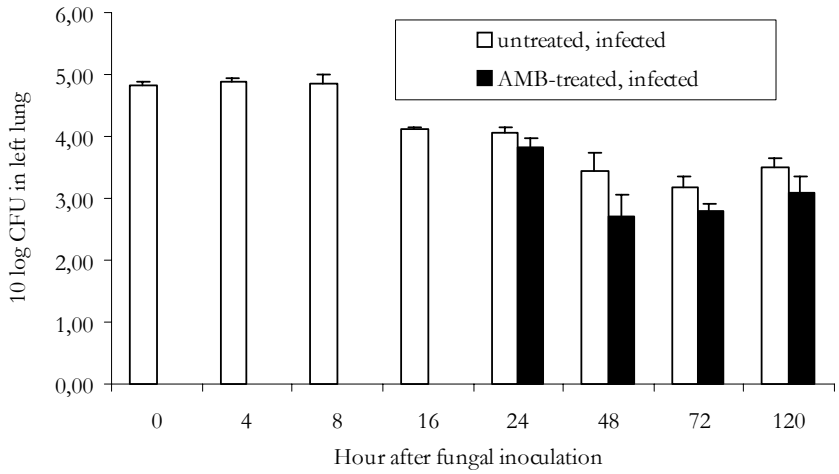


Figure 2. Number of cfu in the infected left lung of rats with IPA. Rats received either treatment with amphotericin B (AMB) 1 mg/kg/day i.v., starting at 16 h after fungal inoculation, or remained untreated. N = 5 to 6 rats per time point.

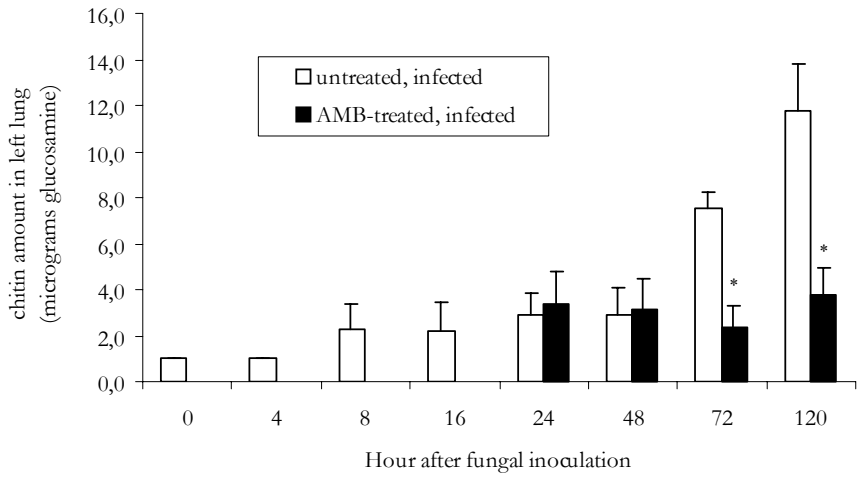


Figure 3. Chitin content of the infected left lung of rats with IPA. N = 5 to 6 rats per time point. See legend Figure 1.

Chitin content

An increase was seen in chitin amount of the infected left lung in untreated, infected rats over time, especially between 48 h and 120 h after fungal inoculation (Figure 3).

Amphotericin B treatment resulted in significant suppression of chitin amounts at 72 h (P = 0.02) and 120 h (P = 0.03) after fungal inoculation. All right lungs were negative for chitin in both groups (data not shown)

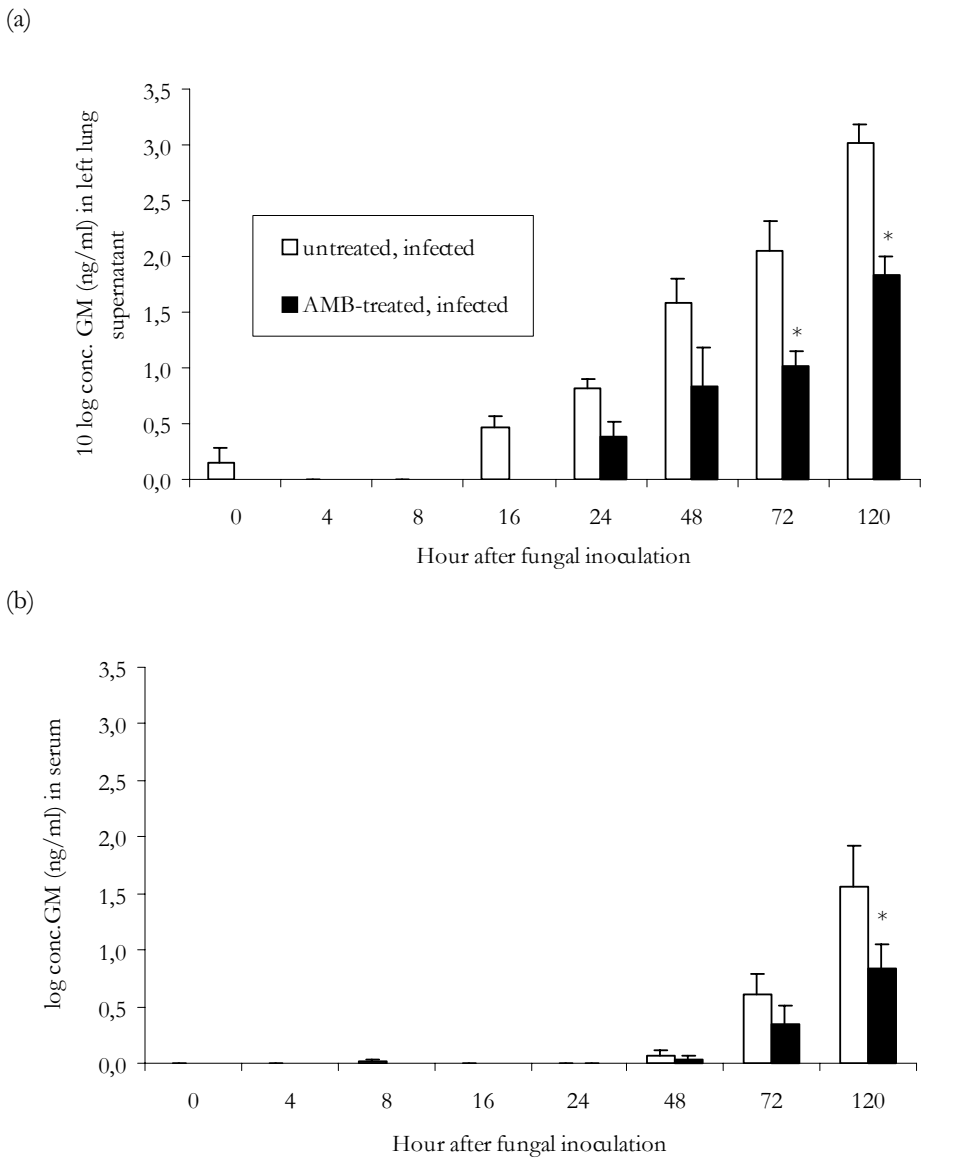
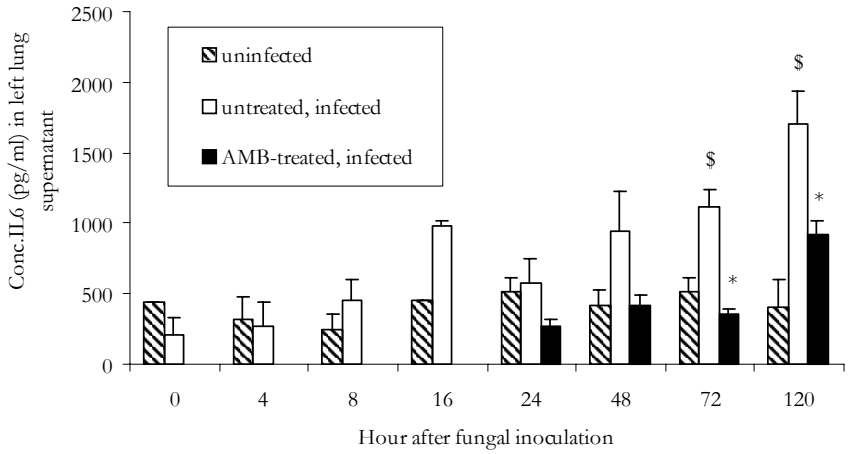


Figure 4. Galactomannan (GM) concentrations in the infected left lung (A) and serum (B) of rats with IPA. N = 5 to 6 rats per time point. See legend Figure 1.

Galactomannan concentrations

Concentrations of galactomannan in the infected left lung increased over time, both in untreated, infected rats and amphotericin B-treated, infected rats (Figure 4). Treatment with amphotericin B suppressed the increase in galactomannan concentrations, with significant differences at 72 h (P = 0.03) and 120 h (P = 0.006). Similar data were seen for galactomannan concentrations in serum, with a significant difference between untreated, infected rats and amphotericin B-treated, infected rats at 120 h (P = 0.04). Galactomannan concentrations in the right lungs of both groups of rats were not significantly elevated compared to uninfected controls (data not shown).

(a)



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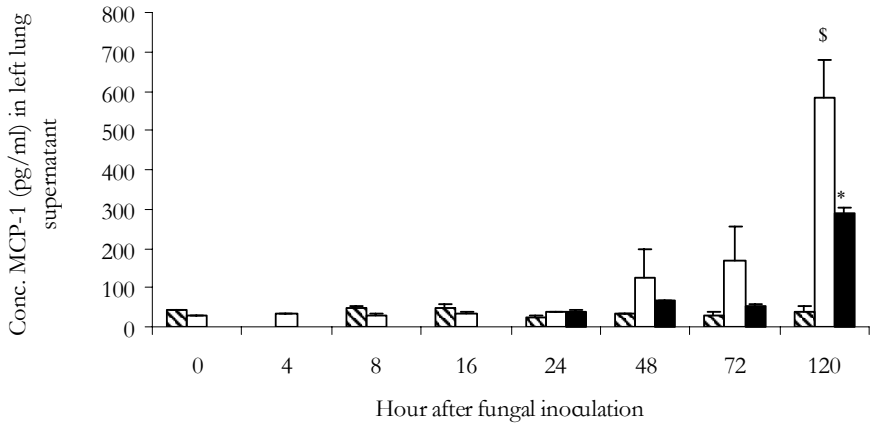

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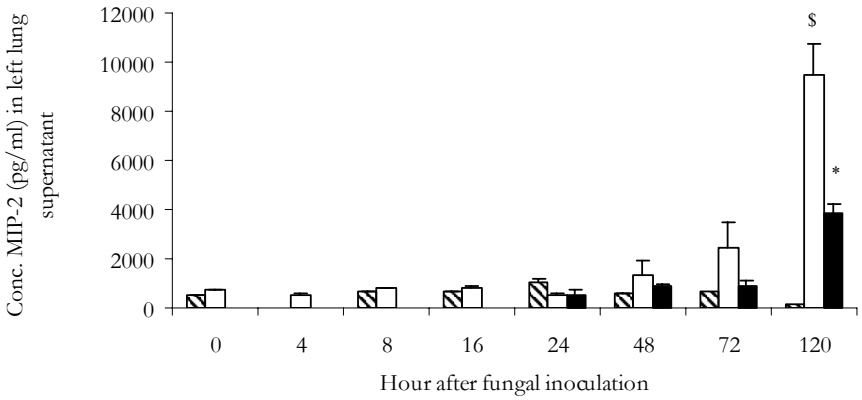

Figure 5. Concentrations of cytokines in left lungs of untreated, infected rats, amphotericin B (AMB)-treated, infected rats and uninfected rats (See also legend Figure 1). N = 5 to 6 rats per time point. * = significant difference compared to untreated, infected rats ($P<0.05$), $ = significant difference compared to uninfected rats ($P < 0.05$).

Concentrations of cytokines

Concentrations of the cytokines TNF-α, IFN-γ, IL-1β, IL-4, IL-6, IL-10, MIP-2 and MCP-1 were measured in the left lung, right lung and serum. The concentrations of TNF-α, IFN-γ, IL-1β, IL-4 and IL-10 did not differ significantly between the three groups of rats. In addition, differences in concentrations between infected left lung and uninfected right lung were not significant in all groups (data not shown). In contrast, IL-6 in the left lung of untreated, infected rats increased over time up to 6- fold compared to uninfected controls (Figure 5A). Amphotericin B - treatment resulted in suppression of increase in left lung IL-6 levels, with significant differences compared to untreated, infected rats at 72 h ($P=0.04$) and 120 h ($P=0.02$). Concentrations of IL-6 in the right lung and serum were not significantly different between the three groups of rats (data not shown). Concentrations of the chemokine MCP-1 in the left lung also increased over time (Figure 5B). Amphotericin B - treatment resulted in lower levels of this chemokine, with a significant difference compared to untreated, infected rats at 120 h ($P = 0.01$). MCP-1 levels in right lung and serum were not different between all three groups of rats (data not shown). Similarly to IL-6 and MCP-1, levels of left lung MIP-2 increased over time, and were suppressed in infected rats receiving amphotericin B - treatment (Figure 5C). The difference between untreated, infected rats and amphotericin B -treated, infected rats was significant at 120 h ($P = 0.02$). Also for this chemokine, concentrations in right lung and serum showed no significant differences between all three groups of rats (data not shown).

DISCUSSION

In a clinically relevant animal model of prolonged severe neutropenic rats, we investigated the pathogenesis of IPA by examining kinetics of parameters of fungal load and cytokines. Standard amphotericin B treatment of established pulmonary infection resulted in survival of only a proportion of rats, which reflects the clinical overall response rate in neutropenic patients with IPA [25]. In untreated, infected rats, the number of left lung cfu did not increase over time despite progression of disease. This is in agreement with the data obtained in several other animal models of IPA [15,16,24,26] In addition, in our model antifungal treatment did not reduce the number of cfu compared to untreated, infected rats, despite a significant increase in survival rate. These observations suggest that the number of cfu is not an adequate measure for quantifying hyphae. Filamentous hyphae composed of multiple cells may only be recorded as a single unit by traditional cfu methodology. Therefore we used other parameters representing the fungal load such as chitin and galactomannan. Chitin content is probably a better measure for fungal load than cfu, since this molecule is a component of the fungal cell wall and in vivo studies have shown that chitin amounts increase with hyphal growth [24,27]. Our study shows that, in contrast to cfu, left lung chitin content increased over time in untreated, infected rats and antifungal treatment resulted in significantly decreased amounts of chitin. We obtained similar results for concentrations of galactomannan, also a fungal cell-wall component, in left lung- and serum. The results indicate that treatment with amphotericin B suppresses increase of fungal load over time in our model, but does not lead to a reverse. Amphotericin B treatment also influenced histopathology of the infected lung. Treatment reduced fungal angio- and broncho-invasion, tissue haemorrhagia and the size of hyphae. Reduction in tissue damage and haemorrhagia and the formation of "resolving lesions" after treatment with amphotericin B has also been described for a rabbit model of IPA [8,28].

Evidence is accumulating that cytokines play a key role in the host response against bacterial as well as fungal infections [29-32]. TNF-α is one of the first cytokines detectable following bacterial or endotoxic challenge, and has been implicated in the cardiopulmonary dysfunction, increased microvascular permeability and metabolic derangements that typify bacteraemic septic shock [33-35]. Increases in TNFα concentrations are also seen in infections with non-bacterial pathogens including rickettsiae, viruses and protozoa. These findings have been taken as evidence for a common host response mechanism involving TNF-α, irrespective of the taxonomic class of the organism [36,37]. In contrast to this hypothesis, levels of TNF-α in serum and infected lungs of rats with IPA in our model were not elevated compared to uninfected controls. Other investigators also found that experimental fungal infection did not result in increased levels of TNF-α. In a systemic rat model of lethal invasive candidiasis serum levels of TNF-α and IL-1α remained low, even at death. These

results were the same for immunocompetent and neutropenic rats. In contrast, when rats were infected with *Escherichia coli* or *Staphylococcus aureus*, large increases of TNF-α and IL-1α were seen [38,39]. These findings suggest that TNF-α is not an essential mediator in lethal fungal infections. The same may be true for IL-1β, since also this cytokine showed no increase in rats with IPA in our model. In contrast, IL-6, another pro-inflammatory cytokine, increased over time in the left lung of rats with IPA in our model. IL-6 can suppress levels of TNF-α, both in human monocytic cell lines and in intact mice [40], and has a function that limits the inflammatory sequelae of TNF and other inflammatory mediators [41]. It is therefore also possible that rapid development of high levels of IL-6 in our model may have down-regulated levels of TNF, and possibly also of IL-1. IL-6 has been shown to play a role in IPA, since IL-6 - deficient mice were more susceptible to IPA than wild-type mice [42], and IL-6 was increased in lungs of immunocompromised mice with IPA compared to uninfected controls [15]. A possible role for IL-6 in IPA might be the induction of granulocyte / macrophage colony-stimulating factor (GM-CSF) expression, which is a molecule known to activate macrophages [43], which in turn are known to be able to kill *Aspergillus* [44].

A number of studies in mice from one group of investigators suggest that survival in IPA was associated with a Th1 response, and succumbing to the infection with a Th2 response [11-14,45]. In the present study the Th1 associated cytokine IFN-γ was not elevated in rats with IPA that survived and the Th2 associated cytokines IL-4 and IL-10 were not elevated in rats that succumbed to the infection. Interleukin-6, which is considered to be a Th2-associated cytokine in murine models [46], was elevated in both treated and untreated rats in our study. Therefore, it could be concluded that in our model, infection with *Aspergillus* resulted in a predominantly Th2 response, that was significantly decreased, but not reversed into a Th1 response as a consequence of amphotericin B treatment. The difference in cytokine profiles between our study and the above-mentioned studies may be explained by the use of a lower inoculum and persistently neutropenic animals in our study, factors that have been shown to have an effect on cytokine concentrations [16,29,47].

In our model we found an increase over time of the left lung levels of the C-X-C chemokine MIP-2, as well as the C-C chemokine MCP-1. These chemokines are produced by a number of cells including macrophages, lymfocytes, endothelial and epithelial cells [43]. Possibly, the production of these chemokines during IPA is a result of endothelial damage during vasal invasion of the fungus and / or direct stimulation by fungal toxins. Elevated levels of these chemokines in animal models of IPA have also been described by others [16,48]. In addition, antibody-mediated neutralization of the C-X-C receptor resulted in an invasive aspergillosis infection, and MCP-1 neutralization decreased conidial clearance in otherwise immunocompetent mice [17,18]. This indicates that these chemokines may play a significant role in the host defense against *A. fumigatus*.

Antifungal treatment of infected rats with amphotericin B in the present model resulted in decreased levels of IL-6, MIP-2 and MCP-1 compared to untreated, infected rats.

Possibly, this could be a direct effect of amphotericin B on the immune system, since this antifungal agent is known to have immunomodulatory characteristics [49]. However, this is not likely since amphotericin B was shown to induce, not suppress, gene expression for TNF-α, IL-1β, MCP-1 and the MIP-2 homologue IL-8 [50-52]. In addition experiments in our laboratory showed that amphotericin B treatment in uninfected rats did not influence cytokine levels in lungs and serum compared to untreated, uninfected rats (data not shown). It is therefore more likely that the decreased cytokine response is a result of the reduction of the fungal load caused by amphotericin B treatment.

From the present study it can be concluded that treatment with amphotericin B reduces the increase in fungal load over time. Probably, this reduction in fungal load results in a decreased local inflammatory response and hence decreased levels of IL-6, MIP-2 and MCP-1 in the infected lung. TNF-α does not play a role in IPA in neutropenic rats. These insights may be useful in the development of new immunomodulatory strategies for the treatment of IPA.

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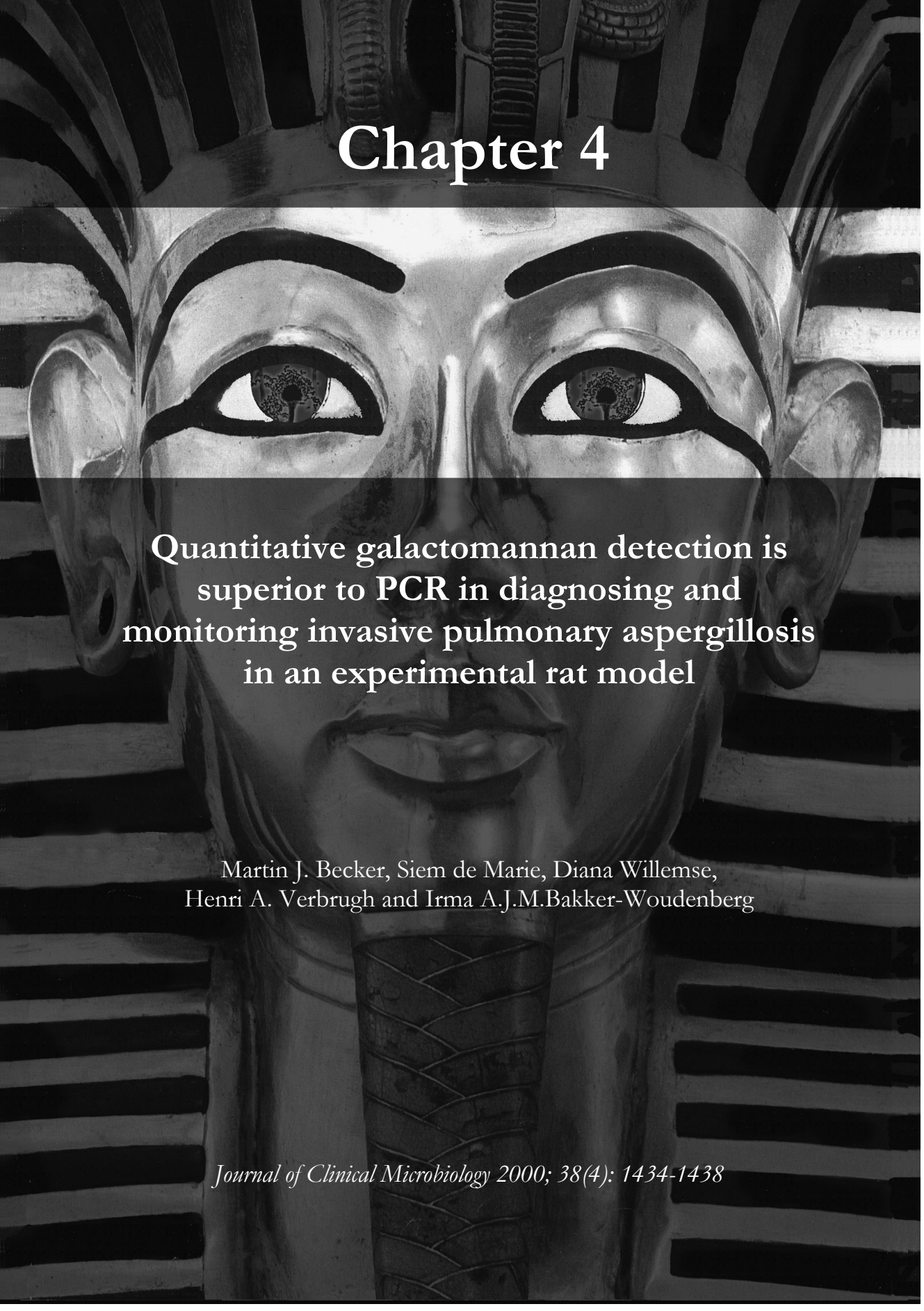

Chapter 4

Quantitative galactomannan detection is superior to PCR in diagnosing and monitoring invasive pulmonary aspergillosis in an experimental rat model

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ABSTRACT

Two diagnostic tests, *Aspergillus*-specific PCR and an ELISA for the quantitative determination of galactomannan, were compared for diagnosing and monitoring invasive pulmonary aspergillosis. Persistently neutropenic rats with left-sided invasive pulmonary aspergillosis were sacrificed at regular intervals after inoculation. Blood samples and BAL-fluid were cultured and tested for PCR as well as ELISA. Disseminated fungal infection to extra-pulmonary organs was determined. Sensitivity of ELISA was higher than that of PCR on all days of measurements, in both blood and BAL-fluid. Positive PCR or ELISA in blood was not significantly associated with disseminated fungal infection. Serial testing in a separate group of rats showed consistently increasing concentrations of circulating galactomannan during the course of disease, while positive PCR could be followed by negative results. The concentration of galactomannan was highly predictive for the time of survival ($P<0.0001$). It was concluded that in this model quantitative galactomannan detection is superior to PCR in diagnosing and monitoring invasive pulmonary aspergillosis.

INTRODUCTION

The incidence of invasive pulmonary aspergillosis (IPA) has increased considerably in the past decade, and this infection is now a major cause of morbidity and mortality in immunocompromised hosts [1]. Patients with prolonged chemotherapy-induced neutropenia and transplant recipients receiving long term, high dose corticoid therapy are at greatest risk [2]. Although mortality rates of IPA remain high despite the use of antifungal therapy, observations suggest that the mortality rate may be reduced by early diagnosis and treatment [3,4]. However, no method has proven sufficiently sensitive and specific to allow a diagnosis at an early stage [5], and new diagnostic methods are therefore under investigation.

Methods for the molecular and serological diagnosis of IPA in blood or broncho-alveolar lavage fluid (BAL-fluid) have especially drawn attention. Using a PCR method for detecting Aspergillus-specific nucleotide sequences, Einsele et al. [6] found a 77% sensitivity in patients with IPA prior to antifungal therapy, increasing up to 100% when two blood samples were analysed. Among the techniques based on antigen detection the sandwich ELISA for the detection of galactomannan (GM) is at the moment the most promising. Studies in neutropenic patients report sensitivities between 70% and 90%, when applying the test in serum [7-9]. It must be noted however, that in these clinical studies it is often not indicated how early during the course of the disease the test becomes positive in relation to the development of clinical and radiological signs. Actually, clinical investigations into sensitivity and specificity of tests for IPA are hampered by the absence of proven infection in many patients and by the fact that the time of onset of infection cannot be determined.

In the present study PCR (two separate assays) and GM detection were evaluated in a rat model of IPA that has been developed in our laboratory [10]. Using this animal model we were able to compare both tests in the early phase of the disease, with a known time of onset of the infection. In addition, we determined the value of these tests for monitoring the course of the disease.

MATERIALS AND METHODS

Infection model of IPA

The animal model was used as described before [10], with some modifications to lengthen the survival time. Specified pathogen free female RP strain albino rats (18-25 weeks old, 185-225 g) were used. Neutropenia was induced by cyclophosphamide (Sigma-Aldrich Chemie, Steinheim, Germany) 75 mg/kg i.p. five days before inoculation, followed by repeated doses of cyclophosphamide 60 mg/kg i.p. at 1 day before and 3 and 7 days after inoculation. This protocol resulted in granulocyte counts of less than 0.1×10^9 / L on the day of inoculation. To prevent bacterial superinfections

animals received ciprofloxacin (660 mg/L) and polymyxin B (100 mg/L) in their drinking water during the whole experiment. Starting 1 day before inoculation, daily i.m. amoxicillin (40 mg/kg/day) was added to this regimen for the remainder of the experiment. On the day of inoculation i.m. gentamìcin (6 mg/kg) was added to the regimen. For infection of the rats a strain of *Aspergillus fumigatus* was used, that was originally isolated from an immunocompromised patient with IPA.
Under general anaesthesia the left main bronchus was intubated. A cannula was passed through the tube and the left lung was inoculated with 2×10^4 *A. fumigatus* conidia. This resulted in a one-sided IPA. Mortality rate was ± 50% on day 7, and 90-100% on day 12 after inoculation. At the end of experiments or at indicated intervals rats were sacrificed and the left lung, as well as the right lung, liver, spleen and brain were homogenised and cultured to determine the presence of disseminated fungal infection. In approximately half of the rats fungal dissemination to extra-pulmonary organs occurred, especially to the liver. Blood cultures for *Aspergillus* species always remained negative in this model.

Blood sampling and broncho-alveolar lavage
Groups of rats were sacrificed to obtain blood for PCR and GM detection and to determine the presence of disseminated fungal infection. Under CO_2 anaesthesia blood samples were taken by cardiac puncture. Broncho-alveolar lavage (BAL) was performed by exposing the trachea and lavaging the lungs 3 times with 5 ml of phosphate-buffered saline (PBS). Of the BAL sample, 2 ml was used for culture, 1 ml for PCR and 300 µl for GM detection. To monitor the course of disease in individual rats, sequential blood sampling was performed by puncture of the orbital plexus.

PCR
Two different methods were used for the extraction of fungal DNA from fluids: the in-house method as developed in our laboratory by van Deventer et al. [11], and the method according to Einsele et al. [6, 12, 13], with some modifications.

DNA extraction from fungal suspensions and BAL-fluid
In the in-house method, 1 ml of fungal suspension or BAL-fluid was centrifuged at 16,000 × g for 5 min. Pellets were resuspended in 0.2 ml of TEG buffer (50 mM glucose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA) containing 1.5 µl lyticase (900 U/ml; Sigma Chemical Co., St. Louise.Mo.) and incubated for 1 h at 37 °C. Subsequently, 3.0 µl of pronase (15 mg/ml; Boehringer GmbH) and 10 µl of 10% sodium dodecyl sulfate (SDS) were added followed by incubation for 1 h at 37 °C. The sample containing fungal DNA was further purified.
In the method according to Einsele, 1 ml of fungal suspension or BAL-fluid was centrifuged (16,000 × g, 10 min) and the pellet resuspended in 0.2 ml of white blood cell lysis buffer (WCLB: 10 mM Tris [pH 7.6], 10 mM EDTA, 50 mM NaCl, 0.2 %

SDS, 200 μg of proteinase K per ml) followed by incubation at 65 ºC for 45 min. After centrifugation (1500 × g, 10 min.) the pellet was resuspended in 0.2 ml zymolyase buffer (50 mM Tris [pH 7.5], 10 mM EDTA, 28 mM β-mercaptoethanol and 300 μg per ml zymolyase [20T ICN, Costa Mesa, California]) and incubated at 37 ºC for 45 min. The solution was centrifuged (1500 × g, 10 min), and the pellet containing fungal DNA was further purified.

DNA extraction from blood specimens

In the in-house method according to van Deventer, 0.5 ml of lysis buffer (0.32 M sucrose, 10 nM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1% Triton X-100) was added to 0.5 ml of EDTA-blood. After lysis, samples were centrifuged (5 min, 16,000 × g) and the supernatant was discarded. The pellet was resuspended in 0.2 ml of lysis buffer. To remove free, non- fungal DNA, 7 μl of DNAse1 (10 mg/ml, Boehringer GmbH, Mannheim, Germany) was added and the samples were incubated at 37 °C for 1 h. After centrifugation at 16,000 × g for 5 min, pellets were resuspended in 0.2 ml of TEG buffer containing 1.5 μl lyticase (900U/ml) and incubated for a further 1 h at 37 °C. Subsequently, 3.0 μl of pronase and 10 μl of 10% SDS were added followed by incubation for 1 h at 37 °C. The sample containing fungal DNA was further purified.

In the method according to Einsele, a volume of 1.5 ml red blood cell lysis buffer (RCLB: 10 mM Tris [pH 7.6], 5 mM $MgCl_2$, 10 mM NaCl) was added to 0.5 ml of EDTA- blood and incubated on a shaking platform for 10 min. The sample was centrifuged (1200 × g, 10 min.) and the pellet was treated again with 1.5 ml of RCLB and centrifuged. Subsequently the pellet was resuspended in 0.2 ml of WCLB and incubated at 65 ºC for 45 min. After centrifugation (1500 × g, 10 min) the pellet was resuspended in 0.2 ml zymolyase buffer and incubated at 37 ºC for 45 min. The sample was centrifuged (1600 × g, 10 min), and the pellet containing fungal DNA was used for further purification.

Purification and amplification of DNA

DNA was purified according to Boom [14]. Briefly, 1 ml of lysis buffer (0.1 M Tris-HCl pH 6.4, 40 mM EDTA pH 8.0, 1 % Triton X-100, 4 M guanidium isothiocyanate) and 50 μl of a Celite suspension (200 mg/ml, Aoroa organics, Grel, Belgium) was added to sample or pellet containing fungal DNA and this suspension was shaken vigorously by hand, followed by incubation at room temperature for 10 min. The suspension was centrifuged (1 min, 15,000 × g) and the pellet was washed two times with a second lysis buffer (0.1 M Tris HCL pH 6.4, 4M guanidium isothiocyanate), two times with ethanol 70 % and one time with acetone, respectively. After drying, the pellet was resuspended in 100 μl of bidestilled water and incubated for 10 min at 56 °C. The sample was centrifuged (15,000 × g, 10 min) and 10 μl of the supernatant was used for amplification.

The following primer set, amplifying a sequence of the multicopy 18S rRNA gene, was used: (5' ATTGGAGGGCAAGTCTGGTG 3') and (5' CCGATCCCTAGTCGGCATAG 3') [6]. PCR was performed in 100 µl of PCR solution containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 2.5 mM $MgCl_2$, 200 µM of (each) dNTP, 50 pmol of each primer, 0.08 U of TAQ polymerase (SuperTAQ, Sphaero Q, Leiden, The Netherlands) and 10 µl sample specimen. Forty cycles of amplification were performed with a PCR processor (9600, Perkin Elmer). Each cycle consisted of a denaturation step at 95 °C for 30 s, a primer-annealing step at 55 °C for 30 s and a chain elongation step at 72 °C for 45 s.

Southern blot analysis of products

20-microliter aliquots of each amplification product were electrophoretically separated on a 1.5% agarose gel in 0.5 × TBE buffer (Tris –borate EDTA). The DNA was transferred from agarose to Hybond-plus nylon filters (Amersham International, Amersham, United Kingdom) by electrophoretic transfer [15]. The PCR-products were analyzed with an Aspergillus specific DNA probe (CATGGCCTTCACTGGCTGTGGGGGGAACCA) [6]. Hybridisation was detected by ECL 3, oligolabeling and detection system (Amersham International).

Sandwich ELISA for detection of GM

The sandwich ELISA was performed as described by Stynen et al. [16] and was used to measure concentrations of GM quantitatively. Some minor modifications were made in the protocol to reduce the standard deviation in series of samples that were spiked with the same concentration of galactomannan. Briefly, 300 µl of each serum or BAL-fluid sample was mixed with 100 µl of treatment solution (4% EDTA) and the mixture was subsequently boiled for 5 min. After centrifugation (20,000 × g, 10 min), the supernatant was used for further testing. Fifty µl of conjugate was added to each well of an anti-galactomannan immunoglobulin M- coated micro-titer plate (Platelia Aspergillus, Sanofi Diagnostics Pasteur), followed by the addition of 50 µl of the treated sample. The plates were incubated at 37 ºC for 90 min. and subsequently washed 5 times with washing buffer (Tris NaCl pH 7.4 containing 1% Tween 20 and 0.01% sodium merthiolate). 200 µl of substrate buffer containing orthophenylenediamine dihydrochloride was added to each well, and the plates were incubated for 30 min at room temperature in darkness. To stop the reaction, 100 µl 1.5 M sulphuric acid was added, and optical density was measured at 450/620 nm. Each plate contained a calibration curve consisting of rat-serum samples containing 0, 1, 1.5, 2, 3, 4, 6, 8 and 12 ng/ml GM (kind gift of Marc Tabouret, Sanofi Diagnostics Pasteur, Steenvoorde, France). A test sample was considered positive when optical density at 450 nm was higher than the cut-off sample (i.e. 1.0 ng). The concentration of GM in positive test samples was expressed as amount of nanograms GM per ml.

Statistical methods
Associations between GM concentrations and PCR results or disseminated fungal infection were analyzed by Mann-Whitney test. Association between PCR-result and disseminated fungal infection was analyzed by chi-square test. Spearman's correlation was used to analyse relations between GM concentrations and time to death.

RESULTS

Validation of two DNA isolation methods
Two methods were used for isolating Aspergillus DNA from blood: an in-house developed method [11], and the method described by Einsele et al. [6], with modifications. The *in vitro* sensitivities of both methods were compared by isolating fungal DNA from blood spiked with 10- fold serial dilutions of *A. fumigatus* conidia. The isolated DNA was then amplified and the amplification product hybridised with an Aspergillus-specific probe. Using both DNA isolation methods, 10 cfu per ml rat-blood could be detected. Sensitivity was not influenced by using larger blood volumes: when volumes of 0.1, 0.5 or 2.5 ml blood were spiked with equal concentrations of conidia, no increase in sensitivity was seen.

Validation of quantitative ELISA
The commercially available "Platelia" sandwich ELISA for detecting GM, was validated for quantitative use in rat serum. Concentrations of 0, 1, 1.5, 2, 3, 4, 6, 8 and 12 ng/ml GM were spiked in six-fold into rat-serum. In Figure 1 the resulting calibration curve is presented. In all samples tested, linear concentration - response curves were obtained between the range of 1 and 8 ng / ml ($r = 0.994$). The detection limit, defined as the concentration corresponding to the mean optical density of the blank + 3 SD, was 1.0 ng / ml.

PCR and ELISA in blood and association with disseminated fungal infection in rats with IPA
Five groups of rats (number varying from 11 to 29) were sacrificed on day 1, 2, 3, 5 and 7 after inoculation (Table 1). Controls (number varying 3 to 9) were inoculated with PBS. From each rat a blood sample was taken for PCR as well as ELISA. The presence of disseminated fungal infection was determined by culture of organs. In all 97 blood samples taken from infected rats, the in- house DNA isolation method was used, and in 68 of these samples in addition the "Einsele" method was used. Both methods showed the same results in 66 samples. Results of the in-house PCR are shown in Table 1. PCR had a considerable lower sensitivity than ELISA, especially between day 2 and 5 after inoculation. Highest rates of positivity for both tests were found on the last day of

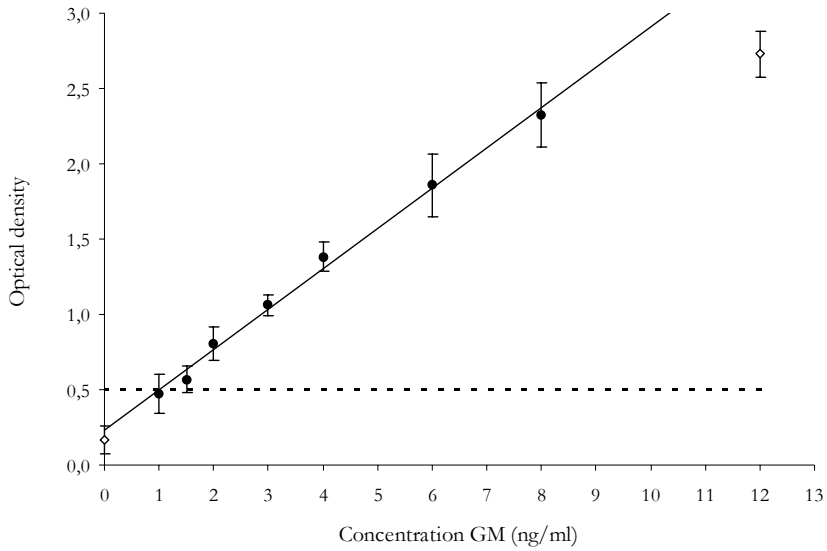

Figure 1. Representative calibration curve of the sandwich ELISA, prepared in 6 - fold with known concentrations of GM in rat serum. Each point represents the mean ± SD (bars). Optical density at 450 nm. The detection limit of the assay is indicated as a discontinuous line.

sampling (day 7): 41 % for PCR and 100% for ELISA. Median concentrations of GM increased from below detection limit in rats on day 1 to 46 ng/ml in rats on day 7. Of all 97 samples, 62% was positive for ELISA; 18% was positive for PCR, all these samples being also positive for ELISA. Specificity was high for both tests: of all 31 blood samples taken from uninfected animals none were found positive for the ELISA, and only one of 31 samples for PCR.

Table 1. PCR and ELISA in blood, and association with disseminated fungal infection in rats with IPA after dissection.

Day	No. Of animals	No. Of animals positive (%)		Median concn (ng/ml) of GM (range)	Dissemination[2)] (no. of rats positive [%])
		PCR[1)]	ELISA		
1	24	0 (0%)	2 (8%)	< 1 (< 1-3)	0 (0%)
2	11	0 (0%)	3 (27%)	< 1 (< 1-7)	0 (0%)
3	18	2 (22%)	16 (89%)	7 (< 1-31)	0 (0%)
5	15	3 (20%)	14 (93%)	19 (< 1-217)	0 (0%)
7	29	12 (41%)	29 (100%)	46 (1.9-600)	11 (38%)

[1)] In -house PCR method

[2)] Extrapulmonary disseminated fungal infection

Results of rats on day 7 were further analyzed to investigate associations between PCR, concentration of GM and the presence of disseminated fungal infection. The median concentration of GM in blood samples that were positive for PCR was higher (48.0 ng/ml, range 8.3 - 602 ng/ml) than the median concentration in PCR-negative samples (30.4 ng/ml, range 1.9 – 480 ng/ml).
Although there was a trend, this difference was not significant (P = 0.09). 37 % of the rats on day 7 showed disseminated infection to extra-pulmonary organs, in all cases the liver was the affected organ. Percentage of rats with positive PCR was higher (57%) in rats with disseminated fungal infection compared to rats with pulmonary infection only (27%). In addition, higher concentrations of GM were found in rats with disseminated fungal infection (median 50.1 ng/ml, range 1.9-602 ng/ml) than without disseminated infection (median 15.8 ng/ml, range 21.0-294 ng/ml). However, both associations were not significant (P-values 0.35 and 0.14 respectively).

PCR, ELISA and fungal culture of BAL-fluid and blood in rats with IPA
Four groups rats (number varying from 5 to 8) were sacrificed on day 1, 3, 5 and 7 after inoculation respectively (Table 2). From each rat blood and BAL-fluid were used for PCR, ELISA and fungal culture. Blood cultures were all negative. Fungal cultures of BAL-fluid were all positive on day 1 after inoculation, the number of cfu decreasing over time. Most BAL-fluid cultures obtained from rats on days 5 and 7 after inoculation remained negative. PCR results were not related to culture findings: one to two positive samples were found on all days. Four rats (two on day 1, one on day 5 and one on day 7) were found negative in blood and positive in BAL-fluid for PCR. ELISA in BAL-fluid was more often positive on day 5 and 7 with increasing titers of GM over time, despite of negative cultures. Three rats (two on day 1, and one on day 5) were negative for ELISA in blood, but positive in BAL-fluid.

Monitoring the course of disease by PCR
Ten rats with IPA were sequentially sampled on day 1, 3, 5 and 7 after inoculation for PCR (Table 3). All rats were negative for PCR on day 1 after inoculation. On day 3, four out of ten rats were positive for PCR, on day 5 two out of eight and on day 7 two out of four. No clear increase in the rate of positive PCR tests was seen during the course of disease. Some rats (1 and 10) remained negative for PCR at all time points, even just prior to death. Two other rats (6 and 8) were positive at day 3, but were found to be negative thereafter.

Table 2. PCR, ELISA and fungal culture of BAL-fluid and blood of rats with IPA after dissection.

BAL-fluid

Day	No. of animals	PCR (no. of rats positive)	ELISA (no. of rats positive)	Median concn (ng/mL) of GM (range)	Culture (no. of rats positive [mean CFU/mL])
1	8	2	2	<1 (<1-2.7)	8 (4)
3	5	1	2	<1 (<1-11)	3 (1)
5	5	2	5	11.6 (5.4-157)	0 (0
7	5	1	5	8.8 (3.9-114)	1 (0)

Blood

Day	No. of animals	PCR (no. of rats positive)	ELISA (no. of rats positive)	Median concn (ng/mL) of GM (range)	Culture (no. of rats positive [mean CFU/mL])
1	8	0	0	<1 (<1-1)	0 (0)
3	5	1	3	4.4 (<1-6.2)	0 (0)
5	5	1	4	10.2 (<1-26.2)	0 (0)
7	5	2	5	19.2 (7.6-48)	0 (0)

Table 3. PCR in blood of 10 individual rats with IPA after sequential sampling

	PCR results on :				
Rat	Day 1	Day 3	Day 5	Day 7	Day 8+
1	-	-	-	Died on day 7	
2	-	-	+	Died on day 7	
3	-	+	Died on day 5		
4	-	-	+	Died on day 7	
5	-	+	+	+	Died on day 8
6	-	+	-	-	Died on day 8
7	-	-	-	+	Died on day 13
8	-	+	-	Died on day 6	
9	-	-	n.d.[1)]	-	Died on day 10
10	-	-	-	Died on day 6	

[1)]not determined

Monitoring the course of disease by GM concentrations

Nine infected rats were sequentially sampled for GM detection (Figure 2). A consistent increase in signal was seen during the course of disease. On day 1 all rats were GM negative, on day 3 all rats were positive for the ELISA and the median concentration of GM was 9.3 ng/ml. On day 5 and 7 median concentrations increased further to 25.8 and 53.0 ng/ml respectively Two rats with relatively high concentrations of GM (71 and 240 ng/ml) on day 5 died the next day. One rat with a relatively low concentration

of GM on day 7 survived relatively long compared to other rats, and died on day 11. We investigated the relation between concentration of GM and time to death. An inverse relation was found that was highly significant (P-value < 0.0001) (Figure 2).

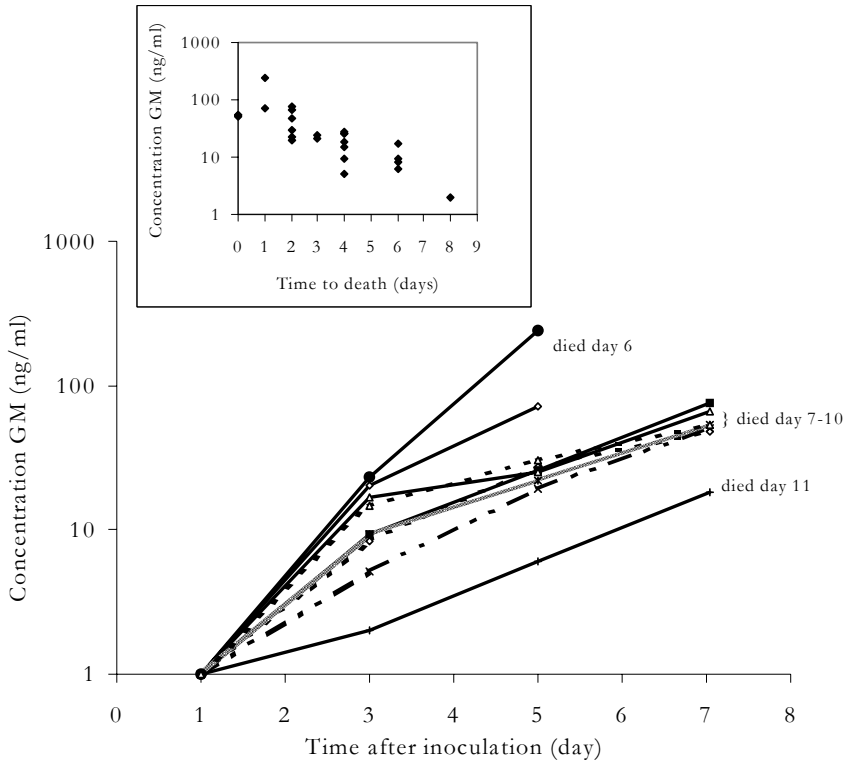

Figure 2. GM in blood of individual rats with IPA after sequential sampling. One line represents one rat. Insert: relation between GM concentrations and time to death.

DISCUSSION

In the present study, we compared two diagnostic tests, Aspergillus-specific PCR and a sandwich ELISA for detecting GM with respect to their value in diagnosing and monitoring IPA in a rat model. Using an animal model, both tests could be evaluated in a controlled fashion in the early phase of the disease, with a known time of onset of infection.

For the diagnosis of IPA in blood, we used two methods for PCR, including the method described by Einsele et al. [6]. These authors found a 77% - 100% sensitivity in patients with IPA. In our model of severe IPA, the maximum sensitivity that was found

using both methods was only 41%, at a moment that more than 50% of the rats had already died (day 7 after inoculation). One explanation for this difference in sensitivity might be the relatively low blood volume (0.5 ml) used for PCR in our model, compared to the blood volume of 3–5 ml used in the study by Einsele et al. These authors suggested in their article that a larger blood volume might help to increase sensitivity of the assay due to the higher yield of fungal DNA. However, when we compared different blood volumes (range 0.1 up to 2.5 ml) obtained from infected rats, we did not observe any increase in sensitivity. Moreover, a higher blood volume may contain more competing DNA or other inhibiting substances which may interfere with the specific PCR signal [17]. In addition, the in vitro sensitivities of both PCR methods we used was 10 cfu per ml, which is similar to the test as described by Einsele et al.

Compared to PCR, the sensitivity of the sandwich ELISA in serum was high in our rat model, up to 100% on day 7. In addition, the ELISA was positive earlier than was the PCR assay. No blood samples from infected animals were PCR positive and ELISA negative. This leads to the conclusion that, at least in our rat model, PCR is not only less sensitive to ELISA but also has no additional value to the ELISA in diagnosis of IPA.

In monitoring the course of disease, PCR showed inconsistent results in the sequentially sampled rats. No clear increase in the fraction of positive animals was seen over time with this assay. Also, an on-off phenomenon was observed: some rats that were positive for PCR, became negative at a later stage in their disease. In the PCR methods we used, fungal DNA was extracted from the pellet obtained after blood cell lysis. Since the pellet contains fungal elements whereas free DNA may be present in the serum, our method could fail to detect circulating free DNA. It is possible that a PCR assay in which circulating DNA is detected in serum gives a better correlation with fungal load and severity of disease, and more consistent results [7, 18]. Comparison of PCR assays in serum with PCR methods as used in our study should be investigated in future studies.

In contrast to PCR in our model, concentrations of GM increased consistently in the course of disease, which has also been reported by others in a rabbit model [19]. In addition, we found a highly significant inverse relation between concentration of GM and time to death of the rats. These findings indicate that concentrations of GM in serum are correlated with the severity of the disease in our model, and possibly with fungal load.

Several other studies have compared PCR and GM detection for the diagnosis of IPA in blood. Hashimoto et al. compared PCR, a (1→ 3) β-D-glucan assay and GM detection in a rat model of IPA [18]. In their study the sensitivity of PCR was higher (80-87%) in the early phase of the disease than that of (1→ 3) β-D-glucan assay (60-75%) and GM detection (71-80%). The same authors found similar results in a study in patients: 70 % sensitivity for PCR and 60 % for GM detection [20]. However, comparison of their findings with our data is difficult, since they used a latex agglutination test for detecting circulating GM, an assay which is about ten times as less

sensitive compared to the sandwich ELISA that we used [9]. Also, the authors used a different PCR method, i.e. a nested PCR in serum. Possibly, a nested PCR is more sensitive than conventional PCR. However, it has been stated that nested PCR is more prone to contamination in a routine hospital laboratory when it is used as a diagnostic tool (16). Bretagne et al. compared GM detection by sandwich ELISA with a PCR in serum of patients with IPA [7]. Similar to our animal study, the authors reported a higher sensitivity for the ELISA compared to the PCR: of the 18 patients with positive mycological data 78% had at least two ELISA-positive sera and 50% had at least one PCR-positive serum. They found only one sample that was positive for PCR and negative for ELISA, and noted that a PCR-positive signal was usually obtained when ELISA was highly positive. This is in accordance with our data: we found no samples that were ELISA negative and PCR positive, and the median concentration of GM tended to be higher in PCR positive samples. Finally, Roth et al. compared PCR by using the method described by Einsele and GM detection by sandwich ELISA in 34 neutropenic patients, of which six with proven IPA [21]. In that study GM detection provided both a higher sensitivity and more consistent results during the course of disease than PCR.

In our model, the yield of fungal cultures of BAL-fluid significantly decreased during the course of disease. Positive results of early cultures were probably related to conidia inoculated into the left lung. In contrast, the GM assay was more often positive later in the disease. Francis et al. found comparable results in a rabbit model of IPA. In their model cultures of BAL-fluid were rarely positive, in contrast to elevated levels of mannitol and GM [22]. These findings are in accordance with results reported by Kauffman et al. [23], who investigated the nature of antigenic determinants released by conidia and hyphae. They found that components that are released spontaneously from conidia are only weakly positive or negative in immunologic assays, in contrast to components released from hyphae. So, it is likely that a strongly immunogenic molecule like GM is released predominantly from hyphae, and in much lesser amounts from conidia. Therefore, GM in BAL-fluid is likely to be a better diagnostic indicator for hyphal growth than routine mycological culture of the organism.

In conclusion, we demonstrated that quantitative GM detection in our model of IPA is superior to PCR, in diagnosing as well as monitoring the disease in both blood and BAL-fluid.

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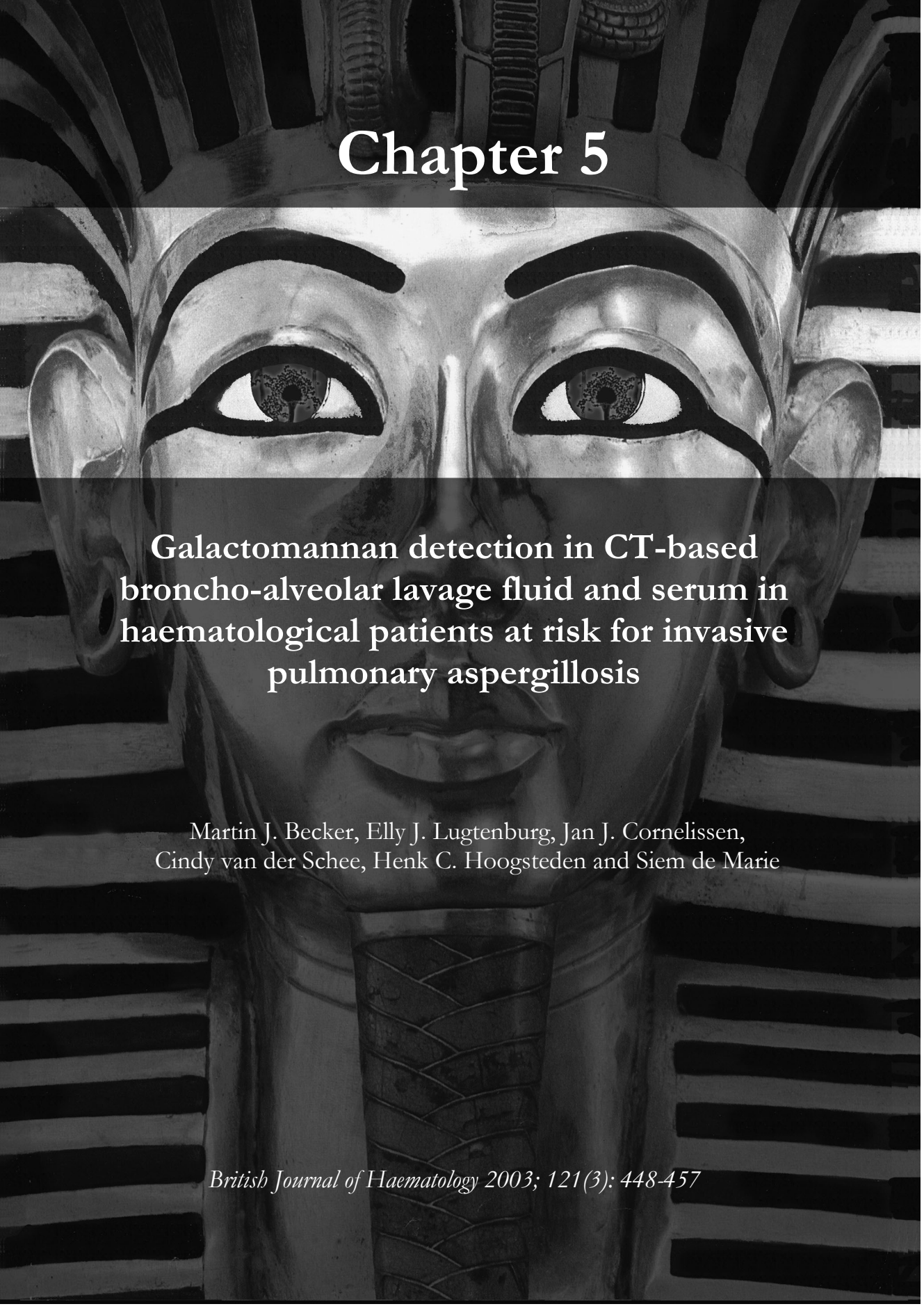

Chapter 5

Galactomannan detection in CT-based broncho-alveolar lavage fluid and serum in haematological patients at risk for invasive pulmonary aspergillosis

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ABSTRACT

We determined the value of galactomannan (GM) detection in CT-based BAL- fluid and serum for the diagnosis of invasive pulmonary aspergillosis (IPA) in haemato-oncologic patients with neutropenia. CT of the thorax and BAL were performed systematically at pre-defined clinical indications. GM was determined by sandwich ELISA, clinicians being unaware of the results. Of 160 patients, 17 (10.6%) presented with proven, probable or suspected IPA. The sensitivity, specificity, positive predicting value (PPV) and negative predicting value (NPV) of GM detection in CT-based BAL-fluid were all 100%. For GM detection in serially sampled serum the sensitivity was 47%, the specificity 93%, the PPV 73% and the NPV 82%. A non-blinded follow-up study was performed to validate the results of GM detection in CT-based BAL-fluid. In this study, 22 of 198 patients (11.1%) presented with IPA and the sensitivity, specificity, PPV and NPV of GM detection in CT-based BAL-fluid were 85%, 100%, 100% and 88% respectively. None of BAL-fluids obtained during antifungal treatment of 3 days ore more were positive. These results indicate that when CT is used systematically and early, GM detection in CT-based BAL-fluid has a high PPV for diagnosing IPA early in untreated patients.

INTRODUCTION

Invasive pulmonary aspergillosis (IPA) remains a major challenge in the management of immunocompromised patients. In neutropenic patients, mortality rates range from 50% to 90 % in different settings [1, 2]. This is probably due to the difficulty in obtaining a reliable diagnosis at an early stage and the relative poor efficacy of the currently available antifungal armentarium [1, 3]. The gold standard for the diagnosis of IPA is the histological demonstration of the fungus in a lung biopsy with concomitant fungal growth from the same specimen. However, biopsies are often precluded by trombocytopenia or by the critical condition of the patient. Therefore, the diagnosis of IPA before death is mostly based on clinical signs, computed tomography (CT)- scan findings and culture of respiratory specimens. The general symptoms -primarily fever refractory to antibacterial therapy, chest pain, cough, and dyspnea- are variable and non-specific. CT of the chest has been advocated for the early diagnosis of IPA as it often shows a "halo-sign" in the early phase of the disease in neutropenic patients with IPA [4-6]. However, the halo-sign is not specific for IPA as it is also seen in a number of other entities including mucormycosis, organising pneumonia and pulmonary haemorrhage [7]. The "air-crescent sign" and other signs of cavitations are highly suggestive for invasive pulmonary fungal infection, but they often appear in a late stage of the disease, after bone marrow recovery. Microscopical examination or culture of respiratory specimens such as broncho-alveolar lavage fluid (BAL-fluid) and sputum have limited sensitivity and do not discriminate between invasive disease, colonisation and contamination [8,9]. Hence, attention has focused on other ways to demonstrate IPA. In this respect, the detection of *Aspergillus* galactomannan (GM) in serum using a commercially available sandwich ELISA [10], has shown promising results [11-13]. The antigen can also be detected in urine [14,15], or BAL-fluid [16-18]. Few studies have compared GM detection in serum and BAL-fluid in neutropenic patients [18,19].
In our institute CT and BAL are routinely used for evaluating haemato-oncological patients at risk for IPA. In this clinical setting we performed a study in two parts. In the first part, we investigated in a blinded study the value of GM detection in serum and BAL –fluid and their relation to CT, fungal culture, histopathology and antifungal treatment. In the second part, GM detection in CT-based BAL-fluid was evaluated as a tool for diagnosing IPA in a non-blinded study.

PATIENTS, MATERIALS AND METHODS

Study population and design

The study consisted of two parts. First, between February 1999 and April 2000 we performed a prospective, blinded study. Thereafter, between June 2000 and October 2001 a prospective, unblinded study was carried out. In both parts of the study haemato-oncologic patients were included that had an expected neutropenia (less than

0.5 x 10^9 cells per litre) for at least 10 days and were 18 years or older. All patients received oral ciprofloxacin (500 mg twice daily) for selective bowel decontamination. In addition, all patients received either fluconazole (200 mg/ day) or itraconazole (200 mg twice daily) as antifungal prophylaxis. During hospitalisation, patients were evaluated for the development of fever and respiratory signs and symptoms. Physical examinations were carried out daily. Chest X-rays were performed at admission and once-twice weekly during hospitalisation, or every other day during periods of fever (T > 38.3 ºC). In case of fever, broad-spectrum antibiotic treatment was administered. Chest CT's were performed when fever of unknown origin lasted for 5 days of antibacterial treatment, or when chest X-rays showed abnormalities. Broncho-alveolar lavage (BAL) was performed if feasible as soon as CT showed abnormalities.

Antifungal treatment was started when new abnormalities emerged on chest X-ray under antibiotic treatment, when abnormalities were found on CT that were compatible with invasive fungal infection, when moulds were cultured from the respiratory tract, when blood cultures revealed fungi or when fever persisted under antibiotics for 7 days.

In the first part of the study, serum samples were taken from all patients twice weekly, starting at the beginning of neutropenia. Sampling was stopped at the end of neutropenia or, in case of (possible) fungal infection, until the end of hospitalisation. Serum and BAL-fluid samples were stored at minus 20 °C. After discharge from the hospital of the patient, the obtained BAL – fluids and serum samples were tested for the presence of GM by a researcher who was unaware of the identity and clinical status of the patient. Clinicians were blinded from the laboratory results in this part of the study.

In the first part of the study, BAL's were performed in about half of the patients with IPA, due to cautiousness of the clinicians to perform the procedure in this fragile group of patients. To exclude selection bias, a second part of the study was performed in which BAL's were performed in all patients with abnormalities on CT unless there was a strong contra-indication. In this unblinded investigation, BAL-fluid samples were tested for GM within three days after the sample was obtained and results were immediately reported back to the clinicians.

The study concerned the routine development of new methodologies in the laboratory and did not involve investigational drugs or additional sampling. Therefore, the study was considered by the ethics committee to be a quality-control investigation of the hospital, and not experimentation with human beings that would require formal ethics review and informed consent. However, all patients signed a declaration that they allowed their clinical data and specimens to be used for research. The first part of the study was blinded and data of galactomannan detection were not used for patient-management. After careful analysis of the data of the first part of the study, CT-based BAL's and galactomannan detection in BAL-fluids were incorporated into the routine work-up of suspected patients, independent of the planning of the second part of the study.

Case definitions and classification

Invasive fungal infections were classified according to the EORTC case definitions, with some modifications [20]. Results of GM detection were excluded from the criteria.

1) Proven invasive pulmonary aspergillosis (IPA) was defined as histopathology or cytopathology showing acutely branched, septated hyphae from a needle aspiration or biopsy with evidence of associated tissue damage (either microscopically or unequivocally by imaging) and a positive culture for *Aspergillus* sp. from sputum or BAL-fluid. The isolation of *Aspergillus* sp. by a sterile procedure: transthoracic or open lungbiopsy / needle aspiration from lungs showing radiological abnormalities consistent with infection was also defined as proven IPA.
2) Probable IPA was defined as a positive culture for *Aspergillus* sp. from sputum or BALF or cytopathology showing acutely branched, septated hyphae together with one major or two minor clinical criteria. Major clinical criteria included (a) halo sign, (b) air-crescent sign or (c) cavitation on CT. Minor clinical criteria included: (a) symptoms of lower respiratory tract infection: cough, chest pain, hemoptysis or dyspnea; (b) physical finding of pleural rub; (c) any new infiltrate not fulfilling a major criterion
3) Suspected IPA was defined as one major clinical criterion together with negative bacterial and fungal cultures from specimens related to lower respiratory tract infection and no evidence for viral disease. Although this category is not included in the EORTC/MSG criteria, there was consensus between clinicians in our department that these patients should be classified as a separate group since they are more likely to have IPA than the patients defined in the "possible" IPA category, and less likely than the "probable" IPA category.
4) Possible IPA was defined as either a) a positive culture or cytology for *Aspergillus* sp., b) at least two minor clinical criteria together with negative bacterial and viral cultures from specimen related to lower respiratory tract infection.
5) Proven and probable other invasive fungal infections (IFI) were defined analogous to the respective IPA categories, with the identification of filamentous fungi by culture or cytology, other than *Aspergillus* sp.

Computed tomography (CT) and classification

CT scans were performed using a Siemens Somaton Plus 4 scan. A scanning protocol was used in which the whole lung fields were scanned with 3-mm thick sections. All CT scans were evaluated after the study by two observers who were unaware of the clinical status of the patients.

Galactomannan (GM) detection

The sandwich ELISA was performed as described by Stynen et al. [10]. Briefly, 300 µl of each serum or BAL-fluid sample was used in sandwich ELISA (Platelia Aspergillus, Sanofi Diagnostics Pasteur, Belgium). Positive and negative controls were included in each assay. All doubtful or positive samples were retested in parallel with recent

samples in the next assay. The OD index was calculated as the OD of the clinical sample divided by the OD of a control sample containing 1 ng /ml GM. As recommended by other authors [12,21], two subsequent serum samples with an index larger than 1.0 were considered positive. A BAL-fluid sample was considered positive when the index was larger than 1.0, as recommended by others [18,22].

Cytology and histopathology
Cytology on BAL-fluids was done by calcofluor white (CFW) stain [23]. Histopathology was done by staining tissue sections with haematoxylin - eosin (H&E) and the other with Grocott's methenamine silver Grocott stain, with haematoxilin-eosin contra-stain [24]

Statistical analysis
We defined the total group of patients with IPA as the sum of patients with proven IPA, probable IPA and suspected IPA. The total group of patients without IPA was defined as the patients that were not classified in any IPA category and did not receive empirical antifungal treatment. Calculations of sensitivity, specificity, positive predictive value (PPV) and negative predicting value (NPV) were based on these two groups.

RESULTS

Study part one:
Comparison of value of GM detection in serum and CT-based BAL-fluid for diagnosing IPA

Patients' characteristics and diagnosis of IPA
Between February 1999 and April 2000 160 patients were included, with a total of 249 neutropenic episodes. The median age of the patients was 49 years (range 18 – 79 years). Patients' characteristics are shown in Table 1. A total of 17 patients (10,6 % ± 2.5 %) were diagnosed as having IPA. Two of these patients had proven IPA, eleven probable IPA and four suspected IPA. In nine patients the causative organism was *Aspergillus fumigatus*, in one patient *Aspergillus flavus* and in one patient *Aspergillus niger*. In two patients acutely branched, septated hyphae were seen in sputum and biopsy indicating *Aspergillus* sp.. In four patients with suspected IPA, clinical and radiological signs were strongly suggestive for IPA, whereas repeated cultures from respiratory specimens remained negative. Six out of 17 patients (35 %) with IPA died during hospitalisation. The cause of death in these patients was pulmonary bleeding due to aspergillosis (3) cerebral aspergillosis (2) and concomitant infection with "*Pneumocystis carinii*" (1). In four patients other invasive fungal infections were diagnosed: *Rhizopus species* (1), *Mucor species* (1), *Saccharomyces cerevisiae* (1) and *Candida kruseï* (1). Hundred seventeen patients did not have a diagnosis of IFI.

CT scan

A total of 475 CT's was performed in all patients, of which 111 CT's in the 17 patients with IPA. Sixteen of 17 IPA patients showed halo-signs on CT, most of them in the earliest stage of disease (Figure 1). In 8 of the 17 IPA patients a crescent or cavitation sign was observed, mostly during bone-marrow recovery. In 15 of the IPA patients multiple nodular- or wedge-shaped lesions were seen, mostly after several weeks of antifungal therapy. The first CT in the neutropenic episode in which the IPA diagnosis was made, showed a halo-sign in 13 patients, multiple nodular lesions in two, non-specific abnormalities in one, and no abnormalities in one patient.

In 74 of 117 patients without IFI, CT showed non-specific abnormalities, which were caused by bacterial or viral infections (27), malignant lymphoma's (24), other (19), or unknown aetiologies (4). A halo sign was seen on CT's of 4 patients, but IFI was discarded because culture or histopathology revealed lymphoma cells (2 patients), influenza (1) and bacterial infection (1). Nodular or wedge shaped lesions were seen in two patients with pulmonary lymphoma. In the remaining 43 patients no CT's were performed or CT's showed no abnormalities.

GM detection in serum

A total of 1145 samples were tested for GM (mean 12.9 samples / patient). Of the 270 samples taken from patients with IPA, 26 (9.6%) were positive, with a median index of 1.891 (range 1.000 - 4.326). Eight of 17 patients with IPA had two or more subsequent sera positive (2 x index ≥ 1.0) for GM. In only one patient with IPA more than three subsequent positive sera were found. In this patient GM was detected for a period of more than 3 months, and finally the antigenemia disappeared and the patient recovered (Figure 1). In the other seven patients the antigenemia was transient, lasting no longer than one week. In six of these seven patients antigenemia disappeared under antifungal treatment, whereas in one patient antigenemia disappeared spontaneously. GM was relatively more often detected in patients that died (4 out of 6, 66%) than in patients that survived (4 out of 11, 36%), but this difference was not significant (P = 0.33). Of 18 patients with possible IPA, 4 patients were GM positive.

Of 44 patients without IFI that were tested for GM, three (7%) were positive. In patient based analysis, the sensitivity of the test using the above criterion was 47 ± 7% (SEM), the specificity 93 ± 5%, the positive predictive value (PPV) 73 ± 6% and the negative predictive value (NPV) 82 ± 5%. When other criteria were used, the results were less favourable (Table 2).

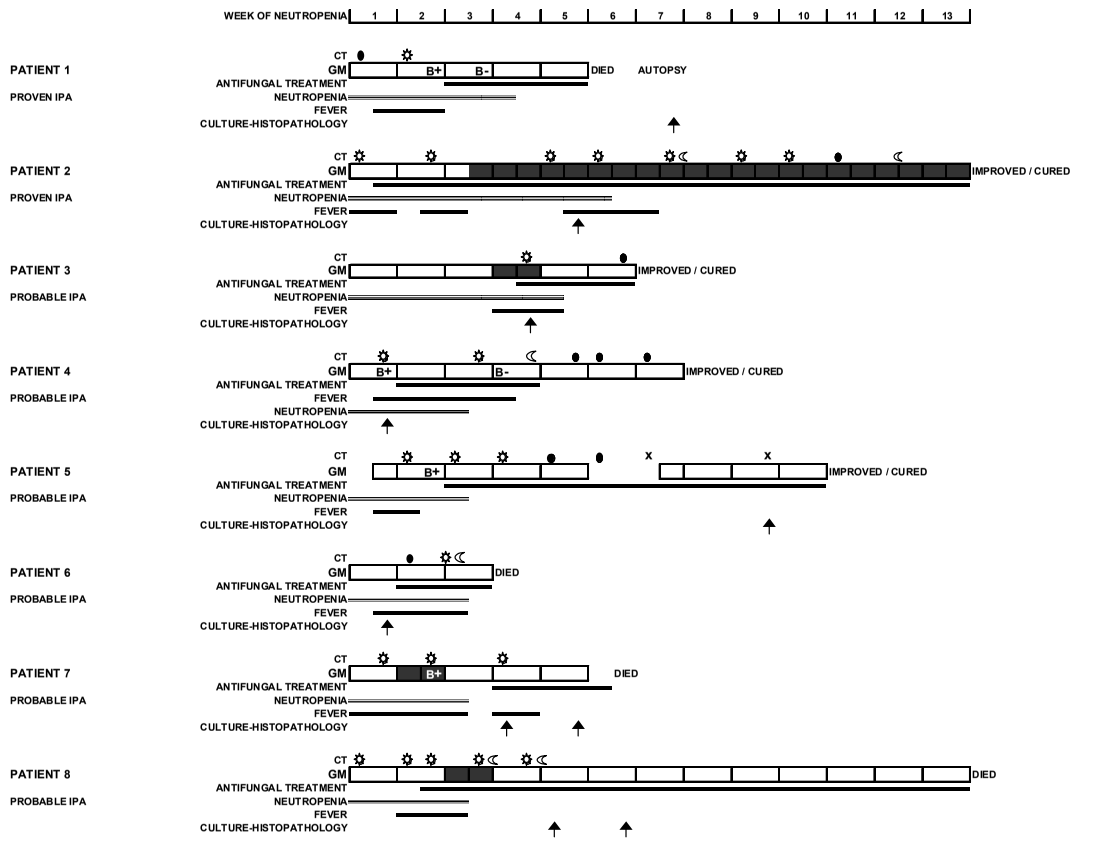
Table 1. Characteristics and serum samples, BAL's and CT distribution of neutropenic patients included in a blinded study between February 1999 and April 2000

	Proven IPA	Probable IPA	Suspected IPA	Possible IPA	Other IFI [1)]	No IFI		Total
						Empiric Ampho B	No Ampho B	
N° of patients	2	11	4	18	4	4	117	160
N° of neutropenic episodes	3	20	6	28	9	6	177	249
Mean duration of neutropenic episode (days)	35	26	32	29	24	22	22	24
Underlying disorder (n° of patients):								
Acute myeloid leukaemia	1	6	3	15	4	2	40	71
Acute lymphoid leukaemia	-	2	-	-	-	-	9	11
Chronic myeloid leukaemia	-	1	-	-	-	1	7	9
Myelodysplastic syndrome	1	1	1	1	-	1	2	7
Non Hodgkin lymphoma	-	1	-	1	-	-	29	31
Hodgkin's disease	-	-	-	-	-	-	5	5
Multiple myeloma	-	-	-	-	-	-	12	12
Other	-	-	-	1	-	-	13	14

Table continued on next page

	Proven IPA	Probable IPA	Suspected IPA	Possible IPA	Other IFI [1)]	No IFI		Total
						Empiric Ampho B	No Ampho B	
Bone marrow transplant (n° of patients):								
Autologic BMT	-	1	-	0	1	1	28	30
Allogenic BMT	-	2	1	3	-	0	30	33
N° of patients with CT's of the chest	2	11	4	18	4	3	77	101
Mean n° of CT's per patient	12	4.9	6.6	3.5	7.3	1.1	1.6	2.1
N° patients with halo / crescent or cavity on CT	2	11	4	-	3	-	4	21
N° of patients with nodular, wedge-shaped or pleura-based abnormalities on CT	2	9	4	18	4	-	2	39
N° of patients with CT's with non-specific abnormalities	-	1	1	5	-	3	74	84
N° of patients with no abnormalities on CT	-	-	1	3	-	2	42	48
N°of patients with BAL's	1	6	2	2	2	1	16	30
Mean n° of BAL's / patient	2.0	0.6	0.5	0.2	0.5	0.3	0.1	0.2
N° of patients tested for serum GM	2	11	4	18	4	4	44	88
Mean n° serum samples / patient	18.5	18.0	15.0	12.8	17.3	14.0	10.4	12.9

[1)] Invasive pulmonary fungal infections other than IPA, proven and probable



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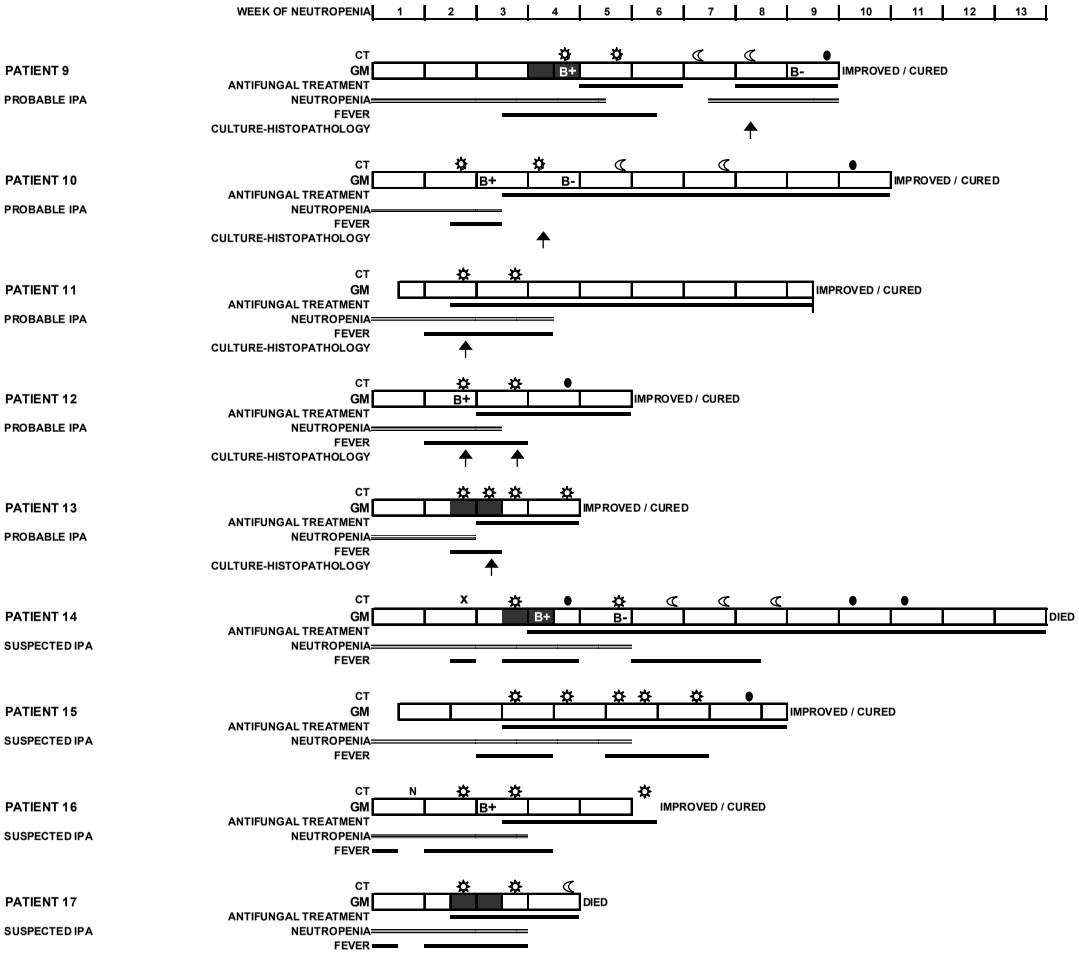
WEEK OF NEUTROPENIA
1
2
3
4
5
6
7
8
9
10
11
12
13
PATIENT 9
PROBABLE IPA
CT
GM
B+
B-
IMPROVED / CURED
ANTIFUNGAL TREATMENT
NEUTROPENIA
FEVER
CULTURE-HISTOPATHOLOGY
PATIENT 10
PROBABLE IPA
CT
GM
B+
B-
IMPROVED / CURED
ANTIFUNGAL TREATMENT
NEUTROPENIA
FEVER
CULTURE-HISTOPATHOLOGY
PATIENT 11
PROBABLE IPA
CT
GM
IMPROVED / CURED
ANTIFUNGAL TREATMENT
NEUTROPENIA
FEVER
CULTURE-HISTOPATHOLOGY
PATIENT 12
PROBABLE IPA
CT
GM
B+
IMPROVED / CURED
ANTIFUNGAL TREATMENT
NEUTROPENIA
FEVER
CULTURE-HISTOPATHOLOGY
PATIENT 13
PROBABLE IPA
CT
GM
IMPROVED / CURED
ANTIFUNGAL TREATMENT
NEUTROPENIA
FEVER
CULTURE-HISTOPATHOLOGY
PATIENT 14
SUSPECTED IPA
CT
X
GM
B+
B-
DIED
ANTIFUNGAL TREATMENT
NEUTROPENIA
FEVER
PATIENT 15
SUSPECTED IPA
CT
GM
IMPROVED / CURED
ANTIFUNGAL TREATMENT
NEUTROPENIA
FEVER
PATIENT 16
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ANTIFUNGAL TREATMENT
NEUTROPENIA
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PATIENT 17
SUSPECTED IPA
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FEVER

Figure 1 ***(previous two pages).*** Timing of CT, GM detection, antifungal treatment , fever and culture/ histopathology in patients with IPA. Symbols: ✲ = halo-sign, ☾ = crescent-sign or cavitation, ⬮ = wedge-shaped or nodular abnormalities, x = non-specific abnormalities. N = no abnormalities. GM detection: □ = serum negative, ■ = serum positive, B+ = BAL-fluid positive, B - = BAL-fluid negative. Arrows: positive findings in culture/ histopathology..

To investigate the inter-laboratory reproducibility of our results, 125 sera taken randomly from patients with IPA, and 75 sera taken from patients with no IFI were retested in an external laboratory (Department of Medical Microbiology, University Medical Center Nijmegen, The Netherlands). The same results were found in 94% of the sera. In the external laboratory, one patient with IPA and one patient without IFI were found positive (2 x index ≥ 1.0) that had been tested negative in our own laboratory. In both cases the disconcordance was based on one serum sample.

Table 2. Sensitivity, specificity and predictive values of GM detection in serum according to different criteria for assessment for positivity.

	Proven, probable and suspected IPA			
Criterion positive	Sensitivity ± SEM	Specificity ± SEM	PPV	NPV
1 x index > 0.7	59%	61%	36%	80%
2 x index > 0.7	53%	89%	64%	84%
1 x index > 1.0	59%	75%	48%	83%
2 x index > 1.0	47%	93%	73%	82%
1 x index > 1.5	18%	84%	30%	73%
2 x index > 1.5	12%	95%	50%	74%

Table 3. GM detection in serum and BAL – fluid in neutropenic patients included in a blinded study between February 1999 and April 2000

		GM detection in serum		GM detection in BAL-fluid	
	Nº patients	Nº patients tested	Nº patients positive (2 x index > 1.0)	Nº patients tested	Nº patients positive (1 x index > 1,0)
Proven IPA	2	2	1 (50%)	1	1 (100%)
Probable IPA	11	11	5 (45%)	6	6 (100%)
Suspected IPA	4	4	2 (50%)	2	2 (100%)
Possible IPA	18	18	4 (22%)	2	1 (50%)
Empiric ampho B	4	4	1 (25%)	1	0 (0%)
Other IFI [1)]	4	4	0 (0%)	2	0 (0%)
No IFI	117	44	5 (11%)	16	0 (0%)

[1)] Invasive pulmonary fungal infections other than IPA, proven and probable

GM detection in CT-based BAL-fluid

In part one of the study a total of 36 BAL-fluids were tested for GM. Of these, 14 BAL-fluids were obtained from nine patients with IPA (see Table 3). In all nine patients with IPA, at least one BAL-fluid was positive for GM (index ≥ 1.0). In all cases this was the first BAL-fluid that was obtained in the neutropenic episode in which IPA was diagnosed (Figure 1). The five BAL-fluids from the patients with IPA that were GM negative were either obtained after one or more weeks of anti-fungal treatment (amphotericin B or lipid formulation of amphotericin B in all cases), or obtained in another neutropenic episode than in the one in which IPA was diagnosed. Of the 14 BAL-fluids obtained from patients with IPA, three revealed a positive culture for *A. fumigatus* and one for *A. niger*. All these four culture positive BAL-fluids were also positive for GM. One BAL- fluid was both positive for CFW and GM. Conversely, four BAL-fluids were positive for GM and both culture and CFW negative. Of the two patients with possible IPA that were tested, one had a BAL-fluid positive for GM.

Nineteen BAL-fluids were taken from 18 patients without IPA. In none of these BAL-fluids GM was detected. In patient based analysis, the sensitivity, specificity, PPV, and NPV were all 100%, using the criterion that at least one BAL-fluid should have an index ≥ 1.0.

Time interval between onset of fever, signs on CT, antifungal treatment and GM detection

In patients with IPA, CT-scans (halo/ crescent or cavitation sign), serum GM, BAL-fluid GM and culture / histopathology were found positive on average 4.0 ± 0.8, 4.6 ± 1.6, 6.9 ± 1.1 and 14.8 ± 5.0 days after onset of fever related to the emergence of IPA respectively. Antifungal treatment was started on average 6 ± 1.4 days after onset of fever.

Study part two:

Prospective investigation to confirm the value of GM detection in CT-based BAL-fluid for diagnosing IPA

Between June 2000 and October 2001 a total of 198 patients were included. The median age of the patients was 47 years (range 18 – 74 years). Twenty-two patients (11.1 %) presented with IPA: three proven, ten probable and nine suspected cases. *A. fumigatus* was the causative organism in eight patients, whereas *A. flavus* was found in the other culture-positive patient with IPA. A total of 453 CT's were performed, of which 95 in 22 patients with IPA. Thirty of these 95 CT's showed a halo-sign, 12 a crescent sign or cavitation and 29 nodular or wedge-shaped lesions.

A total of 80 CT-based BAL-fluids were tested for GM. Of these, 31 BAL-fluids were obtained from 20 patients with IPA. In 17 of these 20 patients at least one BAL-fluid was positive for GM (Table 4). In 16 of these 17 patients, GM was detected in the first BAL-fluid that was obtained in the neutropenic episode in which the IPA diagnosis was

Table 4. GM detection in BAL – fluid in neutropenic patients included in a non- blinded study between June 2000 and October 2001

	GM detection in BAL-fluid		
	Nº patients	Nº patients tested	Nº patients positive (1 x index > 1.0)
Proven IPA	3	3	3 (100%)
Probable IPA	10	9	8 (89%)
Suspected IPA	9	8	6 (75%)
Possible IPA	13	12	5 (42%)
Empiric ampho B	2	2	0 (0%)
Other IFI [1)]	3	2	0 (0%)
No IFI	158	21	0 (0%)

[1)] Invasive pulmonary fungal infections other than IPA, proven and probable

made, in one patient in the second obtained BAL-fluid. In four patients more than one BAL-fluid was positive. However, all BAL-fluids obtained during more than 2 days of antifungal treatment were GM negative. In all these cases antifungal treatment consisted of amphotericin B or lipid formulations of amphotericin B. Of all 21 BAL-fluids from patients with IPA that were positive for GM, only six had positive cultures of *Aspergillus* sp. No BAL-fluids were GM negative and culture-or CFW positive. GM detection was negative in all BAL-fluids of three patients with IPA: one patient with probable and two with suspected IPA. Thirteen patients had possible IPA. A total of 56 CT's were made in these patients, 32 of these showed nodular or wedge-shaped lesions, 21 showed abnormalities that were not suggestive for fungal infection and 3 showed no abnormalities. Twelve of 13 patients had nodular or wedge-shaped lesions on CT at some stage of the disease. GM detection in BAL-fluid was positive in 5 of 12 patients. In 3 of these patients the CT preceding the positive BAL-fluid showed nodular or wedge-shaped lesions, in one patient abnormalities not suggestive for fungal infection and in one patient no abnormalities. Of 12 patients with possible IPA, 5 patients had a BAL-fluid positive for GM. Three patients were diagnosed with other pulmonary fungal infections than IPA: two with *Candida albicans* and one with *Candida krusei* . BAL-fluids taken from these patients were all negative for GM.

A total of 33 BAL-fluids were obtained from 23 patients without IFI. In none of these BAL-fluids GM was detected. In patient-based analysis, the sensitivity of the test was 85 ± 8% SEM, the specificity and PPV were 100% and the NPV was 88 ± 7%, using the criterion of at least one BAL-fluid with an index ≥ 1.0.

Combined calculation of values of GM detection in BAL-fluid in part one and two of the study

Analysing the combined data of all 108 patients included between February 1999 and October 2001 in which one or more BAL's were performed, the sensitivity for GM

detection in CT-based BAL-fluid was 90 ± 5.6%, the specificity and PPV were 100% and the NPV 93 ± 4%.

DISCUSSION

The diagnosis of IPA remains a difficult task, especially in neutropenic patients. Early diagnosis is of great importance since early start of antifungal treatment improves survival [25]. CT is recently recognised as an important tool for diagnosing IPA. Caillot *et al.* suggested that a systematic use of CT scan allowed earlier diagnosis of IPA and significantly improved survival when combined with early antifungal therapy [4]. However, the use of CT scan seems to differ between different centres. For example, in some studies CT's were performed in only a minority of patients with IPA [22,26], whereas in the study by Caillot et al. CT's were performed in all of 25 patients with IPA, with a mean of almost 3 CT's per patient [4]. In our study CT's were performed systematically and early in all patients with IPA, with a mean of 5.3 CT's per patient and starting within four days after onset of fever.

GM detection for diagnosing IPA has been investigated in several studies [11-14,17,18,22,27,28]. The antigen can be detected by a latex agglutination test or by a sandwich ELISA, of which the latter has shown to have the higher sensitivity [28,29].

Recently, several large, prospective studies in neutropenic patients reported sensitivities, specificities and predictive values above 90% for GM detection by ELISA in serum [11-13]. In our study these characteristics were less favourable: we found a sensitivity of GM detection in serum of 47%, a specificity of 93%, a PPV of 73% and a NPV of 82%. Differences in definitions of IPA may partly explain this disparity. Another reason for the observed difference may be that early and systematic use of CT in our study results in less advantageous characteristics of the test. Early detection of suspected lesions on CT might have resulted in an earlier start of antifungal treatment. Since antifungal treatment has been shown to suppress circulating GM levels [27], and we observed that serum GM often converted to negative as soon as antifungals were started, early start of treatment may have prevented detectable antigenemia in a number of patients. With a PPV of 73% and a NPV of 82%, GM detection in serum may still have some additional value for diagnosing IPA in the individual patient in our clinical setting. However, these values were only achieved when serum was sampled serially twice per week, and using the optimal criterion of two subsequent indexes ≥ 1.0.

In a very recent, large study in oncological patients also less favourable test characteristics of GM detection in serum were reported, i.e. a sensitivity of 32%, a specificity of 95%, a PPV of 58% and a NPV of 85%. In this report the presence in patients of circulating anti-Aspergillus antibodies was suggested as a possible cause for the relatively low sensitivity and PPV [30].

In contrast to GM detection in serum, we found a surprisingly high sensitivity (100%) and specificity (100%) for GM detection in CT based BAL-fluid in the first part of our

study. To confirm these figures a second investigation was started, in which BAL's were performed more frequently. Overall, the sensitivity of GM detection in CT based BAL-fluid was 90%, the specificity 100%, the PPV of 100% and NPV of 93%. However, these figures were based on BAL-fluids obtained before start of antifungal treatment. All BAL-fluids obtained under more than two days of antifungal treatment, and most BAL- fluids obtained after ceasing antifungal treatment were negative. This indicates that BAL for GM detection should be performed early, promptly after CT and before the start of antifungal treatment. Possibly, the use of antifungal treatment could explain the somewhat lower sensitivities of GM detection in BAL-fluid found in other studies, in which use of antifungals before or during BAL was not reported [17,18]. In accordance with our findings, Caillot et al. found higher sensitivities for GM detection in BAL fluid (73%-83%) than in serum (41-45%) using the latex agglutination test, in a clinical setting with early and systematic CT use [4,5].
A number of studies have reported the use of PCR in BAL-fluid for diagnosing IPA [17,31-33]. However, the PPV of this test for diagnosing IPA may be compromised by the fact that this test does not distinguish between infection and colonisation [31,32]. This problem may be less when GM detection is used, as is suggested by the high PPV in our study. In addition, in our animal model of IPA it was shown that GM detection in BAL-fluid was more often positive during invasive fungal growth than during colonisation, in contrast to PCR [34].
We also investigated the time interval between onset of fever, first halo-sign on CT, antigen detection and culture / histopathology in the first part of our study. CT was found positive already 4 days after onset of fever and GM was detected in BAL fluid after 7 days. In comparison, culture / histopathology became positive only 15 days after onset of fever on average. This indicates that a diagnostic strategy in which GM detection in CT based BAL is used, results in an earlier confirmed diagnosis of IPA compared to classical methods.
We conclude that, when CT is used early and systematically, GM detection in CT-based BAL fluid has a high PPV for diagnosing neutropenia-associated IPA in an early phase of the disease. BAL-fluids should be obtained before start of antifungal treatment, since under antifungal treatment GM detection becomes negative. GM detection in serum seems to be of less value, since its sensitivity and PPV are limited.

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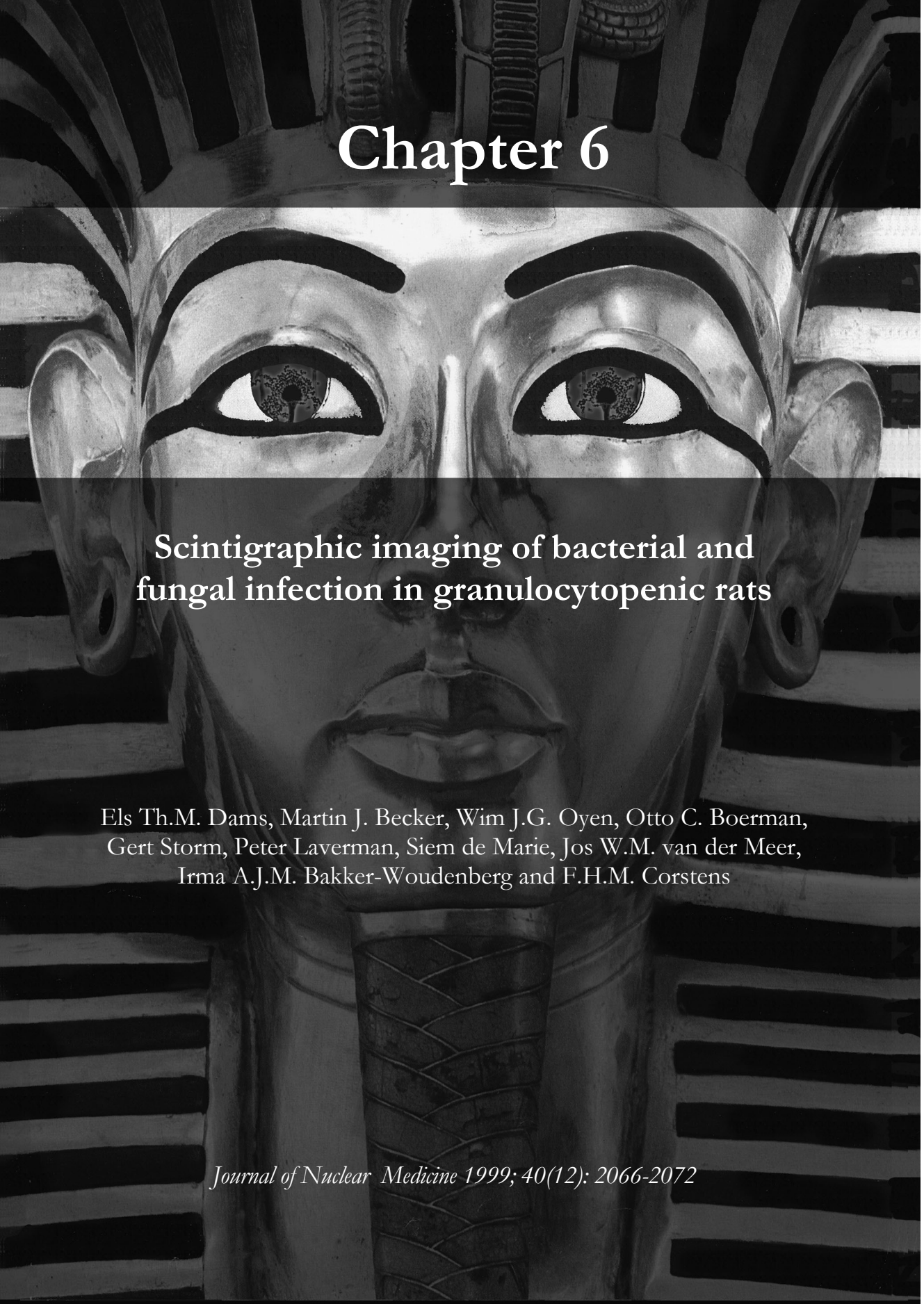

Chapter 6

Scintigraphic imaging of bacterial and fungal infection in granulocytopenic rats

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ABSTRACT

Scintigraphic imaging in granulocytopenic patients can be very useful to detect and localize infections, which often do not show localizing signs and symptoms. We studied the potential of ^{99m}Tc-labeled polyethylene glycol (PEG)-coated liposomes and ^{99m}Tc-labeled IgG to image bacterial and fungal infection in a granulocytopenic rat model. ^{67}Ga-citrate was used as a reference agent.

Methods. ^{99m}Tc-PEG-liposomes, ^{99m}Tc-hydrazinonicotinate(HYNIC)-IgG or ^{67}Ga-citrate were administered to granulocytopenic rats with a *Staphylococcus aureus* abscess or with unilateral invasive pulmonary aspergillosis. Imaging and biodistribution studies were performed.

Results. All agents visualized the *Staphylococcus aureus* infection from 1 h postinjection onwards. However, only with ^{99m}Tc-PEG-liposomes and with ^{99m}Tc-HYNIC-IgG activity in the infectious foci increased with time up to 24 h. ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG showed significantly higher accumulation in the infectious focus as compared to ^{67}Ga-citrate (1.33 ± 0.31 %ID/g and 1.40 ± 0.16 %ID/g, respectively, vs. 0.31 ± 0.04 %ID/g; 24 h p.i., $p<0.05$). At 24 h p.i. abscess-to-muscle ratios were highest for ^{99m}Tc-liposomes (72.1 ± 19.1), followed by ^{99m}Tc-HYNIC-IgG (18.3 ± 3.3) and ^{67}Ga-citrate (4.4 ± 0.7). In pulmonary aspergillosis, both ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG showed significantly higher uptake in the infected lung than ^{67}Ga-citrate (3.6 ± 0.4 and 8.3 ± 0.8 %ID/g, respectively, vs. 1.3 %ID/g at 24 h p.i.; $p<0.05$).

Conclusion. ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG performed better than ^{67}Ga-citrate in the localization of peripheral bacterial infection and fungal infection in the lung in granulocytopenic rats. The high focal uptake and high target-to-nontarget ratios of ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG indicate that both radiopharmaceuticals may become valuable agents to image infection in granulocytopenic patients.

INTRODUCTION

Febrile episodes frequently occur in the treatment of cancer patients, particularly during the severe and often protracted neutropenic period induced by aggressive chemotherapeutic regimens. In granulocytopenic patients, more than 60 % of these febrile episodes are of infectious origin, causing substantial morbidity and mortality [1]. Prompt initiation of empiric broad-spectrum antibiotic therapy has shown to improve clinical outcome [2]. Rapid identification of a possible site of infection helps to tailor the instituted antibiotic regimen and reduces the occurrence of side-effects, of superinfection and the emergence of resistant micro-organisms. However, granulocytopenic patients often lack localizing signs or symptoms, hampering clinical identification of an infectious focus [3]. Scintigraphic techniques can be useful in the diagnostic process as they provide rapid whole-body evaluation based on functional processes, while they do not depend on morphological abnormalities. Imaging with labeled autologous leukocytes is very effective for detection of acute infection [4,5], but is obviously not feasible in granulocytopenic patients. Labeled donor leukocytes have been proposed as an alternative method [6,7], but are only seldomly applied because of the high risk for HLA-immunization, transfusion-associated graft-versus-host disease, and transmission of viruses [8,9]. Therefore, gallium-67-citrate (^{67}Ga-citrate) is currently the method of choice for scintigraphic evaluation of the febrile granulocytopenic patient [10,11]. Opportunistic respiratory infections in particular are adequately visualized with this radiopharmaceutical [11]. However, imaging with ^{67}Ga-citrate has several disadvantages: (1) the radiopharmaceutical has unfavourable imaging characteristics and causes high radiation exposure, (2) the physiological bowel uptake and the accumulation in malignant lymphomas limits its accuracy for the detection of (abdominal) infections, (3) optimal imaging often requires delayed recordings up to 72 hours, whereas a timely diagnosis may have a major clinical impact in these patients.
Several new agents are being developed for infection imaging. Nonspecific polyclonal human immunoglobulin labeled with indium-111 has shown high efficacy for visualizing focal infection in both immunocompetent and granulocytopenic patients [12,13]. A ^{99m}Tc-label would be more attractive, providing better image quality and lower radiation exposure. IgG labeled with ^{99m}Tc via the nicotinyl hydrazino derivative (HYNIC) has shown high accuracy for the detection of clinical infection and inflammation [14], but has not yet been studied in the granulocytopenic host. Sterically stabilized liposomes, also called PEG-liposomes, are another newly developed agent in the field of scintigraphic detection of inflammation and infection. The inclusion of polyethyleneglycol (PEG) into the lipid bilayer of these vesicles results in prolonged blood circulation time compared to conventional liposomes, due to reduced uptake by the mononuclear phagocyte system [15,16]. Experimental studies have shown excellent targeting of inflammatory foci with PEG-liposomes [17-19].
In the present study, we evaluated the potential of ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG to visualize bacterial infection in granulocytopenic rats. In addition, the

performance of labeled liposomes and labeled IgG was evaluated in a clinically more relevant model of pulmonary aspergillosis, a frequent and serious fungal infection in immunocompromised patients. For comparison, ^{67}Ga-citrate was included as a reference agent.

MATERIALS AND METHODS

Infection models

Staphylococcus aureus. A focal *S. aureus* abscess was induced in granulocytopenic rats, as described by Oyen et al. [13] with minor modifications. Briefly, male randomly-bred Wistar rats, weighing 200-220 g, were injected with one dose of cyclophosphamide (Asta-Medica, Diemen, The Netherlands) 100 mg/kg intraperitoneally five days before bacterial inoculation (day –5), followed by one dose of 75 mg/kg on day -3. The rats had free access to standard rat chow and acidified drinking water. The degree of granulocytopenia was tested in a separate group of five rats. The mean WBC count was reduced from 11.3 ± 0.5 x 10^9/l on day -5 to 0.2 ± 0.1 x 10^9/l on day 0, and remained on this level until the end of the experiment. Differential counts showed that less than 20% of the leukocytes were granulocytes, resulting in a granulocyte count of < 0.05 x 10^9/l. Under ether anaesthesia, a focal abscess was induced by injecting approximately 1 x 10^6 viable *S. aureus* (ATCC 25923) in 0.1 ml 50%:50% suspension of autologous blood and normal saline in the left calf muscle. Twenty-four hours after the inoculation, when swelling of the muscle was apparent, the respective radiopharmaceuticals were injected via the tail vein.

Pulmonary aspergillosis. Pulmonary aspergillosis was induced in granulocytopenic rats according to the methods described by Leenders et al. with minor modifications [20]. Briefly, female RP strain rats, 18-25 weeks old and weighing 185-225 g, were injected with one dose of cyclophosphamide 75 mg/kg intraperitoneally five days before fungal inoculation (day -5), and one dose of 60 mg/kg on day -1. This protocol resulted in granulocyte counts of less than 0.1 x 10^9/l on the day of inoculation until the end of the experiment. To prevent bacterial superinfections, animals received ciprofloxacin (660 mg/L) and polymyxin B (100 mg/L) in their drinking water during the whole experiment. Daily intramuscular amoxillin injections (40 mg/kg/day) were added to this regimen starting one day before inoculation. Pulmonary infection was established, using a strain of *Aspergillus fumigatus* isolated from an immunocompromised patient with invasive pulmonary aspergillosis. 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 2 x 10^4 *Aspergillus fumigatus* conidia in 0.02 ml suspension of phophate buffered saline (PBS, pH 7.4). This procedure resulted in a left-sided invasive pulmonary infection; signs of mycelial disease were usually seen at 16 h after inoculation [20]. Three rats were inoculated with 0.02 ml normal saline and served as controls.

Radiopharmaceuticals

^{99m}Tc-PEG-liposomes. Glutathione-containing PEG-liposomes were prepared as described previously [19]. The mean size of the liposome preparations as determined by dynamic light scattering measurements was 100-110 nm with a polydispersity index < 0.1. Preformed glutathione-containing liposomes were labeled with ^{99m}Tc essentially as previously described [21]. Briefly, the liposomes (70 μmole phospholipid/ml) were incubated for 15 min at room temperature with freshly prepared ^{99m}Tc-HMPAO (6 MBq/μmole phospholipid). Labeling efficiency was between 70 and 80%. Removal of unencapsulated ^{99m}Tc-HMPAO was achieved by gel filtration on a PD-10 column (Pharmacia, Woerden, The Netherlands) with 5% glucose as the eluent. Radiochemical purity as determined on a gelfiltration column (PD-10) was higher than 95%.

^{99m}Tc-HYNIC-IgG. Hydrazinonicotinamide (HYNIC) was synthesized and conjugated to human polyclonal IgG (Gammagard, Baxter/Hyland, Lessines, Belgium) according to the methods of Abrams and colleagues [22]. Approximately one HYNIC group was coupled per IgG molecule, as determined spectrophotometrically. The purified HYNIC-conjugated IgG was diluted to 4 mg/ml in 0.15 M acetate (pH 5.85), sterilized by membrane filtration and stored at -20 ^{0}C in 0.5 ml aliquots. After thawing 0.5 ml HYNIC-IgG-solution, the conjugate was radiolabeled with ^{99m}Tc by adding 0.1 mg N-[Tris(hydroxymethyl)-methyl]glycine (Tricine, Fluka), 0.01 mg $SnSO_4$, and ^{99m}Tc pertechnetate (50 MBq/mg). The mixture was incubated for 15 min at room temperature. The radiochemical purity was determined by instant thin layer chromatography on silica gel strips (Gelman Lab., Ann Arbor, MI) with 0.15 M acetate (pH 5.85) as the mobile phase. Labeling efficiency was always higher than 95%. HPLC analysis on a size exclusion column indicated that the preparation contained less than 5% aggregates, as described previously [30].

^{67}Ga citrate.. ^{67}Ga-citrate (DRN 3103) was purchased in kit form (Mallinckrodt Medical, Petten, The Netherlands).

Study design

S. aureus abscess. Twenty-four hours after bacterial inoculation, groups of three rats were injected via the tail vein with either 10 MBq ^{99m}Tc-PEG-liposomes or 10 MBq ^{99m}Tc-HYNIC-IgG or 10 MBq ^{67}Ga-citrate. The animals were anesthetized with a mixture of halothane, nitrousoxide and oxygen, and were placed prone on a single head gamma camera equipped with a parallel-hole, low-energy collimator for the ^{99m}Tc studies or a medium-energy collimator for the ^{67}Ga studies. Each group of rats was imaged at 5 min and 1, 2, 4, 10 and 24 hr after injection. Images (300,000 counts/image; at 24 hr p.i. 100.000 counts/image) were obtained and stored in a 256 x 256 matrix. The scintigraphic results were analyzed by drawing regions of interest over the abscess, the noninfected contralateral calf muscle (used as background region), the heart (representing blood-pool activity) and the whole animal. Abscess-to-background ratios and the percentage residual activity in the abscess (abscess-to-whole body ratio) were

calculated at various timepoints. The ex-vivo biodistribution of the radiolabels was determined in a separate experiment. Twenty-four hours after bacterial inoculation, groups of five rats were injected intravenously via the tail vein with either 4 MBq ^{99m}Tc-PEG-liposomes or 4 MBq ^{99m}Tc-HYNIC-IgG or 0.4 MBq ^{67}Ga-citrate. Twenty-four hours after injection of the radiopharmaceutical, the rats were killed with 30 mg phenobarbital injected i.p.. Blood was obtained by cardiac puncture. After cervical dislocation, tissue samples (right calf muscle, infected left calf muscle, lung, spleen, kidney and liver) were dissected, weighed and their activity was measured in a shielded well-type gamma counter (Wizard, Pharmacia-LKB, Sweden). To correct for physical decay and to calculate uptake of the radiopharmaceuticals in each tissue sample as a fraction of the injected dose, aliqouts of the injected dose were counted simultaneously. The results were expressed as percent injected dose per gram (%ID/g). Abscess-to-blood and abscess-to-muscle ratios were calculated.

Pulmonary aspergillosis. Forty-eight hours after inoculation, groups of six infected rats were injected intravenously via the tail vein with either 10 MBq ^{99m}Tc-PEG-liposomes or 10 MBq ^{99m}Tc-HYNIC-IgG or 10 MBq ^{67}Ga-citrate. The non-infected control rats received 10 MBq ^{99m}Tc-PEG-liposomes. Imaging studies were performed as described above. The rats were imaged at 2, 8 and 24 h after injection. After obtaining the final image, the rats were killed to determine the ex-vivo distribution of the radiolabels. After assessment of macroscopic abnormalities, small samples of the right and left lung were collected for microbiological examination. The remainders of the right and left lung were weighed and their activity was measured in a shielded well-type gamma counter. Tissue samples of other organs were processed as described above. Results were expressed as percent injected dose per gram (%ID/g). Left-to-right lung, left lung-to-blood and left lung-to-muscle ratios were calculated.

Microbiological and histopathological studies

S. aureus abscess. S. aureus infection was induced in two granulocytopenic rats and two immunocompetent rats, as described above. Samples of infected left calf muscle and noninfected right calf muscle were dissected, formalin-fixed and embedded in paraffin. Sections were cut and stained with hematoxylin-eosin for lightmicroscopic examination.

Pulmonary aspergillosis. Samples of the right and left lung were aseptically removed. A sample was smeared on Colombia III agar blood plates (Becton & Dickinson, Etten-Leur, The Netherlands) which were incubated at 37 ^{0}C for 48 h to examine possible bacterial co-infection. The remainders of the lung tissues were fixed in buffered formalin. After measurement of radioactivity, the tissues were embedded in paraffin and histopathological studies were done on sections that were stained with hematoxylin-eosin or Grocott methenamine silver.

Statistical analysis

All values are given as percent injected dose per gram tissue (%ID/g) or ratios ± one standard error of the mean (SEM). Statistical analysis of tissue distribution was

performed using the one-way-analysis-of-variance (ANOVA). Tukey-Kramer multiple-comparison tests were applied. A corrected p value of < 0.05 was considered significant.

RESULTS

S. aureus abscess. Although a relatively small inoculum was used (1 x 10^6 viable bacteria in granulocytopenic rats *vs* 1 x 10^9 viable bacteria in immunocompetent rats), gross swelling of the left calf muscle was apparent in all granulocytopenic rats 24 hours after bacterial inoculation. Cross-sections of the abscess samples showed an encapsulated collection of purulent material. No haemorrhages were observed. Microscopic examination revealed necrosis of muscle tissue, but only minimal leukocyte infiltration in contrast to the massive leukocyte influx seen in immunocompetent rats (Figure. 1). Figure 2 shows the scintigraphic images of the respective radiopharmaceuticals at 1 and 24 hours postinjection. All agents visualized the abscess as early as 1 hr postinjection. Furthermore, improving contrast between abscess and background on the subsequent scintigrams was observed with the ^{99m}Tc-labeled agents, but not with ^{67}Ga-citrate. Quantitative analysis of the images showed that abscess uptake of ^{67}Ga-citrate only slightly increased with time and reached a maximum of 6.8 ± 1.2 %ID at 10 hr postinjection. The abscess-to-background ratio at 24 h postinjection did not exceed 2.0 and was significantly lower than that of the two other agents ($p<0.01$). In contrast, both ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG showed increasing accumulation in the abscess throughout the 24 hour period, resulting in significantly higher abscess uptake (16.6 ± 1.9 %ID and 16.3 ± 1.6 %ID, respectively) and abscess-to-background ratios (16.6 ± 1.1 and 12.8 ± 1.4, respectively), as compared to ^{67}Ga-citrate ($p<0.05$). Blood clearance of ^{67}Ga-citrate was significantly faster

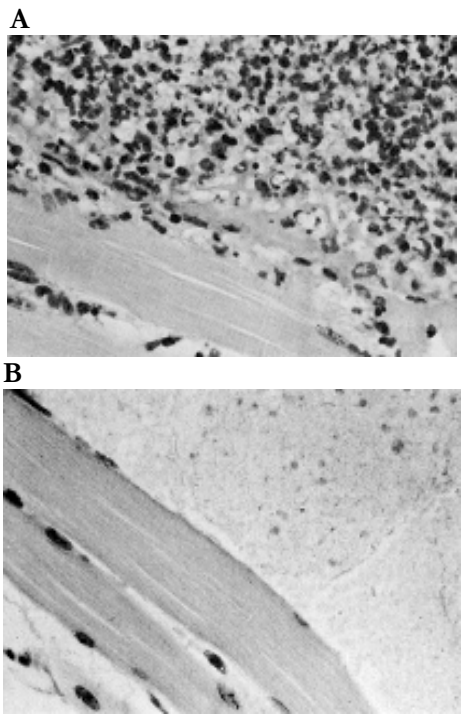

Figure 1. Hematoxylin-eosin stained tissue of S. aureus-induced abscess in immunocompetent rats (A) and granulocytopenic rats (B). Note the massive leukocyte infiltration in A, not present in B. Magnification x 400.

than that of the ^{99m}Tc-labeled compounds ($p<0.01$ at 1 hr p.i.; data not shown): the initial $t_{½}$ was approximately 1.5 hr compared to approximately 6 hr and 10 hr for the ^{99m}Tc-liposomes and ^{99m}Tc-HYNIC-IgG, respectively.

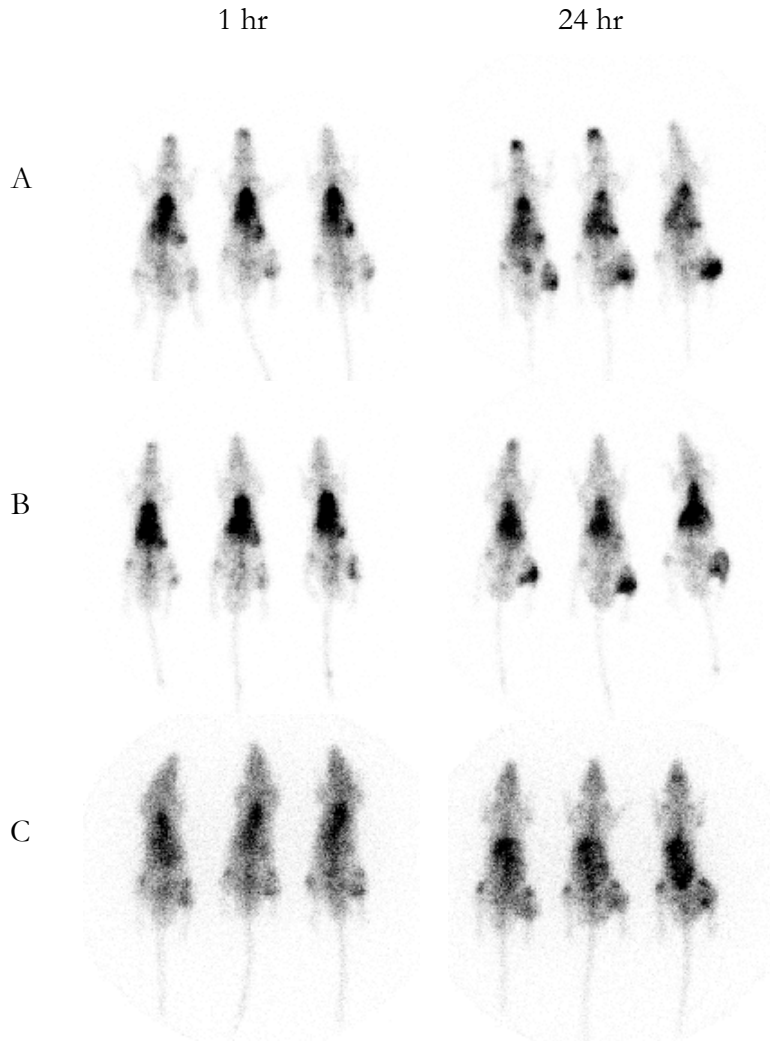

Figure 2. Scintigrams of granulocytopenic rats with S. aureus infection in the left calf muscle imaged at 1 h and 24 h after injection of ^{99m}Tc-PEG-liposomes (A), ^{99m}Tc-HYNIC-IgG (B) and ^{67}Ga-citrate (C).

Table 1. Biodistribution of ^{99m}Tc-PEG-liposomes, ^{99m}Tc-HYNIC-IgG and ^{67}Ga-citrate in granulocytopenic rats with S. aureus abscess 24 hr p.i. (%ID/g ± SEM)

Organ	Liposomes	IgG	Ga-67
Blood	0.87 ± 0.06	1.22 ± 0.25	0.07 ± 0.004
Muscle	0.02 ± 0.001	0.08 ± 0.01	0.07 ± 0.01
Abscess	1.33 ± 0.31	1.40 ± 0.16	0.31 ± 0.04
Lung	0.32 ± 0.02	0.72 ± 0.12	0.14 ± 0.01
Spleen	5.75 ± 0.74	2.11 ± 0.16	2.30 ± 0.46
Kidney	2.53 ± 0.20	1.27 ± 0.04	0.44 ± 0.03
Liver	0.58 ± 0.07	1.06 ± 0.05	0.64 ± 0.03
Abscess/blood	1.57 ± 0.41	1.29 ± 0.27	4.71 ± 0.66
Abscess/muscle	72.1 ± 19.1	18.3 ± 3.3	4.40 ± 0.71
Abscess/spleen	0.22 ± 0.03	0.68 ± 0.09	0.16 ± 0.04
Abscess/liver	2.28 ± 0.37	1.32 ± 0.10	0.49 ± 0.07

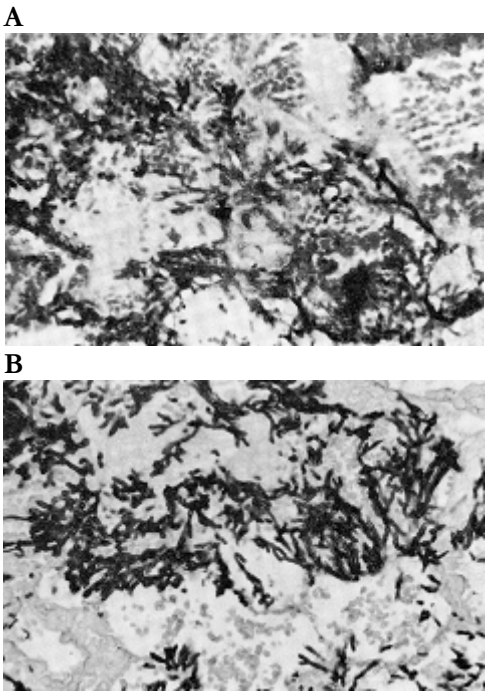

Figure 3. Lung tissue of granulocytopenic rats with invasive pulmonary aspergillosis showing abundant septate hyphae of Aspergillus with only minimal cellular infiltration. (A) Hematoxylin-eosin stain. (B) Grocott methenamine silver stain. Magnification x 300.

The biodistribution data of the radiolabels following injection of the three radiopharmaceuticals are given in Table 1. In accordance with the scintigraphic results, ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG showed significantly higher uptake in the infectious focus at 24 hr postinjection than ^{67}Ga-citrate ($p<0.05$). The very low uptake of the labeled liposomes in normal muscle resulted in an abscess-to-muscle ratio of 72.1 ± 19.1, four to sixteen times higher than the ratios obtained with ^{99m}Tc-HYNIC-IgG ($p<0.05$) and ^{67}Ga-citrate ($p<0.01$). Due to the relatively low blood level of ^{67}Ga-citrate, the abscess-to-blood ratio of gallium was higher than for the other two agents ($p<0.01$). Biodistribution of the three radiopharmaceuticals in the various organs showed relatively high splenic uptake with ^{99m}Tc-PEG-liposomes (5.75 ± 0.74 %ID/g; $p < 0.01$), while liver uptake was highest with ^{99m}Tc-HYNIC-IgG (1.06 ± 0.05 %ID/g; $p < 0.001$).

Pulmonary aspergillosis. Gross haemorrhagic infarctions were macroscopically visible at the base of the infected left lung. In the IgG-group, the infarctions appeared to be more extensive than in the other two groups (mean affected lung surface 25% vs. 15%), but the difference was not statistically significant. Histological examination revealed abundant septate branching hyphae invading blood vessels and lung tissue, but only minimal leukocyte infiltration (Figure 3). No mycelial disease was observed in right lung tissue or in lung tissue of the control rats. Negative microbiological findings excluded bacterial co-infection. The scintigraphic images of the radiopharmaceuticals are shown in Figure 4. At 24 hr p.i., increased uptake in the left lung could be noted with ^{99m}Tc-liposomes and ^{99m}Tc-HYNIC-IgG. At earlier time-points, pathologic uptake in the region of the left lung could not unequivocally be observed since adequate delineation of the infection was hampered by the relative small size of the animals and the vicinity of activity in the cardiac pool. With ^{67}Ga-citrate, minimal uptake was noted in the left lung at all time points. No increased pulmonary uptake was observed in the control rats, who had been injected with labeled PEG-liposomes. Quantitative analysis of the images revealed that both ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG displayed relatively long half-lives; the absolute values were remarkably higher than in the *S. aureus* experiment (initial $t_{½}$ approximately 20 and 30 hr, respectively, vs. 6 and 10 hr; data not shown).

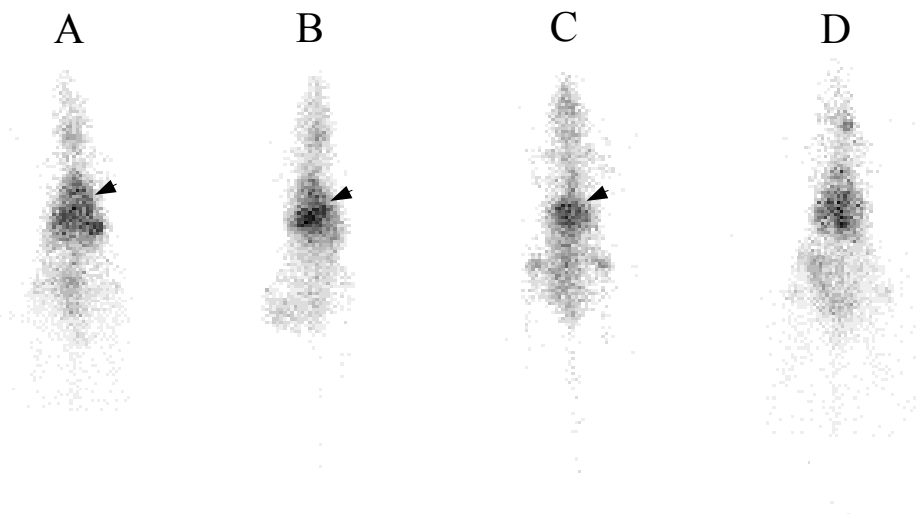

Figure 4. Scintigraphic images of granulocytopenic rats with invasive pulmonary aspergillosis 24 hr after injection of ^{99m}Tc-PEG-liposomes (A), ^{99m}Tc-HYNIC-IgG (B) or ^{67}Ga-citrate (C). (D) Non-infected control rats, injected with ^{99m}Tc-PEG-liposomes, 24 hr p.i.. Arrows indicate pathological uptake in the left lung.

The biodistribution data of the three radiopharmaceuticals, given in Table 2, show that uptake in the infected lung was significantly higher with ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG than with ^{67}Ga-citrate ($p<0.05$ and $p<0.001$, respectively). Due to the relatively high uptake of labeled IgG in normal lung tissue, the liposomes displayed the highest left-to-right lung ratio (3.4 ± 0.4) of the two ^{99m}Tc-agents, and had also a higher ratio than ^{67}Ga-citrate (1.1 ± 0.1). Furthermore, the left lung-to-muscle ratio was significantly higher with the liposomes (96.6 ± 17.1) than with ^{67}Ga-citrate (21.1 ± 5.5; $p<0.001$). The left lung-to-blood ratio was similar for the three radiopharmaceuticals. The biodistribution of labeled liposomes in normal lung tissue was highly similar for rats with or without lung infection. The lung uptake in non-infected tissue was higher than observed in the *S. aureus* experiment.

Table 2. Biodistribution of ^{99m}Tc-PEG-liposomes, ^{99m}Tc-HYNIC-IgG and ^{67}Ga-citrate in granulocytopenic rats with pulmonary aspergillosis 24 hr p.i. (%ID/g ± SEM)

Organ	Liposomes (asp)	IgG (asp)	Ga-67 (asp)	Liposomes (contr)
blood	2.89 ± 0.25	5.90 ± 0.42	0.65 ± 0.11	2.74 ± 0.97
muscle	0.04 ± 0.01	0.15 ± 0.01	0.07 ± 0.02	0.13 ± 0.02
left lung	3.59 ± 0.44	8.31 ± 0.80	1.30 ± 0.36	1.13 ± 0.38
right lung	1.08 ± 0.10	3.03 ± 0.28	0.55 ± 0.16	1.02 ± 0.38
spleen	10.60 ± 1.54	7.30 ± 0.73	1.62 ± 0.40	13.28 ± 2.55
kidney	2.58 ± 0.30	3.21 ± 0.71	2.04 ± 0.34	3.00 ± 0.18
liver	1.38 ± 0.13	4.18 ± 0.46	1.85 ± 0.35	1.49 ± 0.15
left/right lung	3.4 ± 0.4	2.8 ± 0.3	2.4 ± 0.4	1.1 ± 0.1
left lung/muscle	96.6 ± 17.1	58.2 ± 6.1	21.1 ± 5.5	10.5 ± 5.3
left lung/blood	1.2 ± 0.1	1.4 ± 0.1	2.2 ± 0.7	0.4 ± 0.02

asp = *Aspergillus* infection; contr = control.

DISCUSSION

The paucity of diagnostic clues in the febrile granulocytopenic patient and the limitations of current radiopharmaceuticals stimulate the search for agents capable to rapidly and effectively visualize infectious foci, not depending on the patients' leukocyte count and/or immune status. Our study shows that ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG can localize focal bacterial infection in granulocytopenic rats. Compared to ^{67}Ga-citrate, ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG had the highest absolute abscess uptake (up to 1.40 %ID/g). A very high abscess-to-muscle ratio (>70) was observed with ^{99m}Tc-PEG-liposomes, exceeding the values of all other agents. The relatively high uptake of the ^{99m}Tc-PEG-liposomes is in line with our observations in previous studies [23], and most likely is due to physiological filtration rather than

phagocytosis by spleen macrophages [24]. In addition to high focal uptake in bacterial infection, labeled PEG-liposomes and labeled IgG also showed preferential accumulation in lung tissue infected with *Aspergillus*. Uptake with these ^{99m}Tc-labeled agents (up to 8.3 %ID/g) was significantly higher than with ^{67}Ga-citrate (1.3 %ID/g). Furthermore, target-to-nontarget ratios exceeded the ratios obtained with ^{111}In-IgG in rats with Pneumocystis carinii pneumonia (PCP), another opportunistic respiratory infection [19]. As ^{111}In-IgG as well as ^{67}Ga-citrate have proven their clinical usefulness for the detection of these type of infections [25], the results of our study hold promise for the clinical application of ^{99m}Tc-labeled liposomes and ^{99m}Tc-HYNIC-IgG for the detection of aspergillus infection. The excellent targeting of pulmonary aspergillosis with labeled liposomes is remarkable, since this agent failed to localize experimental PCP [19]. Apparently, the causative microorganism and its histopathological features affect preferential localization. Invasive pulmonary aspergillosis in granulocytopenic subjects typically shows abundant hyphae and hemorrhagic infarctions with virtual absence of neutrophilic and monocytic lesions [26,27], whereas tissue damage is less prominent in PCP and a mononuclear infiltrate prevails [28]. Perhaps in PCP the relatively large size of liposomes prevents adequate extravasation compared to the the smaller IgG molecules.

Currently, a series of radiolabeled peptides (f-MetLeuPhe, interleukin-1, interleukin-1 receptor antagonist, interleukin-2, tuftsin, leukotriene, platelet factor 4, and others) is tested for their ability to image infection and inflammation scintigraphically [29]. It is expected that this approach will eventually lead to a ^{99m}Tc-labeled agent for infection imaging that will allow rapid and specific localization of infection and inflammation. However, these peptides are directed against receptors expressed on cells that infliltrate in inflammatory foci and their localization relies on cellular infiltrates. As we have shown here, in neutropenic subjects only minimal leukocyte infiltration may occur.

The uptake in the abscess and the abscess-to-muscle ratio of both ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG were higher than found in previous studies in immunocompetent rats with focal infection [19,30]. This may be related to the stronger inflammatory response seen in neutropenic animals; it has been shown that the proinflammatory cytokine response in neutropenic mice is stronger than in immunocompetent mice [31], which may have led to relatively more endothelial damage and vascular leakage. In addition, a stronger outgrowth of bacteria could have occurred in the immunocompromised animals. Still, the results are remarkable, as we had to use a relatively low dose of *S. aureus* to avoid overwhelming sepsis and acute mortality (10^6 viable bacteria in granulocytopenic rats *vs.* 10^9 viable bacteria in immunocompetent rats). Obviously, no such comparative data are available for pulmonary aspergillosis. In the *Aspergillus* experiment, the radiopharmaceuticals showed higher uptake in non-infected tissue than in the *S. aureus* experiment, which was probably due to differences in breed.

The results of our study confirm that localization of labeled IgG and liposomes in infectious foci does not depend on interaction with inflammatory cells, as both

infection models displayed a conspicious absence of leukocytes, while focal uptake in *S. aureus* abscess was at least as high as in immunocompetent animals. Indeed, several studies have indicated that labeled liposomes as well as labeled IgG extravasate into inflammatory areas just by virtue of locally enhanced vascular permeability [32,33]. Thus, the blood level of the radiopharmaceutical is considered to be the driving force for accumulation at the site of infection. Consequently, the improved infection targeting of PEG-liposomes compared to that of conventional liposomes is attributed to their enhanced blood circulation time [34].

The moderate performance of ^{67}Ga-citrate in both infection models is remarkable, as previous studies have shown the usefulness of ^{67}Ga-citrate for the localization of infectious foci in immunocompromised patients [11]. However, these studies were mainly performed in patients who were not deeply neutropenic. Although the mechanism of ^{67}Ga-citrate concentration in areas of inflammation is still subject to debate, it is suggested that lactoferrin present in polymorphonuclear leukocytes binds to ^{67}Ga and thus contributes to focal accumulation [35]. This concept could possibly explain the performance of ^{67}Ga-citrate in our granulocytopenic rat model.

In view of the histopathological findings in pulmonary aspergillosis, it is conceivable that besides increased vascular permeability, focal bleeding may have contributed to focal uptake of labeled liposomes and labeled IgG. This could have biased the results in favour of IgG given the more extensive hemorrhagic infarctions in the IgG group. The contribution of focal bleeding to the uptake of the two agents could be of importance to determine their potential role in the diagnostic management of *Aspergillus* infection. Although the incidence of infarctions has no relation with the amount of viable microorganisms and bleeding can occur early in *Aspergillus* infection [27], further studies are needed to evaluate the performance of labeled liposomes and IgG in relation to duration and severity of the disease.

If application under granulocytopenic conditions is feasible, ^{99m}Tc-labeled agents are preferred over ^{67}Ga-citrate – currently the agent of choice for opportunistic infections in immunocompromised patients – because of lower radiation exposure, more favourable imaging characteristics and greater availability. In addition, the higher focal uptake of ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG, and the higher target-to-nontarget ratios compared to ^{67}Ga-citrate, suggest improved infection targeting in granulocytopenic patients when these agents are employed.

CONCLUSION

In a granulocytopenic rat model, ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG provided good visualization of *S. aureus* induced inflammation early after i.v. injection, and showed high focal uptake and target-to-nontarget ratios compared to ^{67}Ga-citrate. In granulocytopenic rats with pulmonary aspergillosis, ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG showed significantly higher uptake than ^{67}Ga-citrate. These results

indicate that ^{99m}Tc-PEG-liposomes and ^{99m}Tc-HYNIC-IgG might be useful in the evaluation of the febrile granulocytopenic patient, and therefore warrant further clinical studies.

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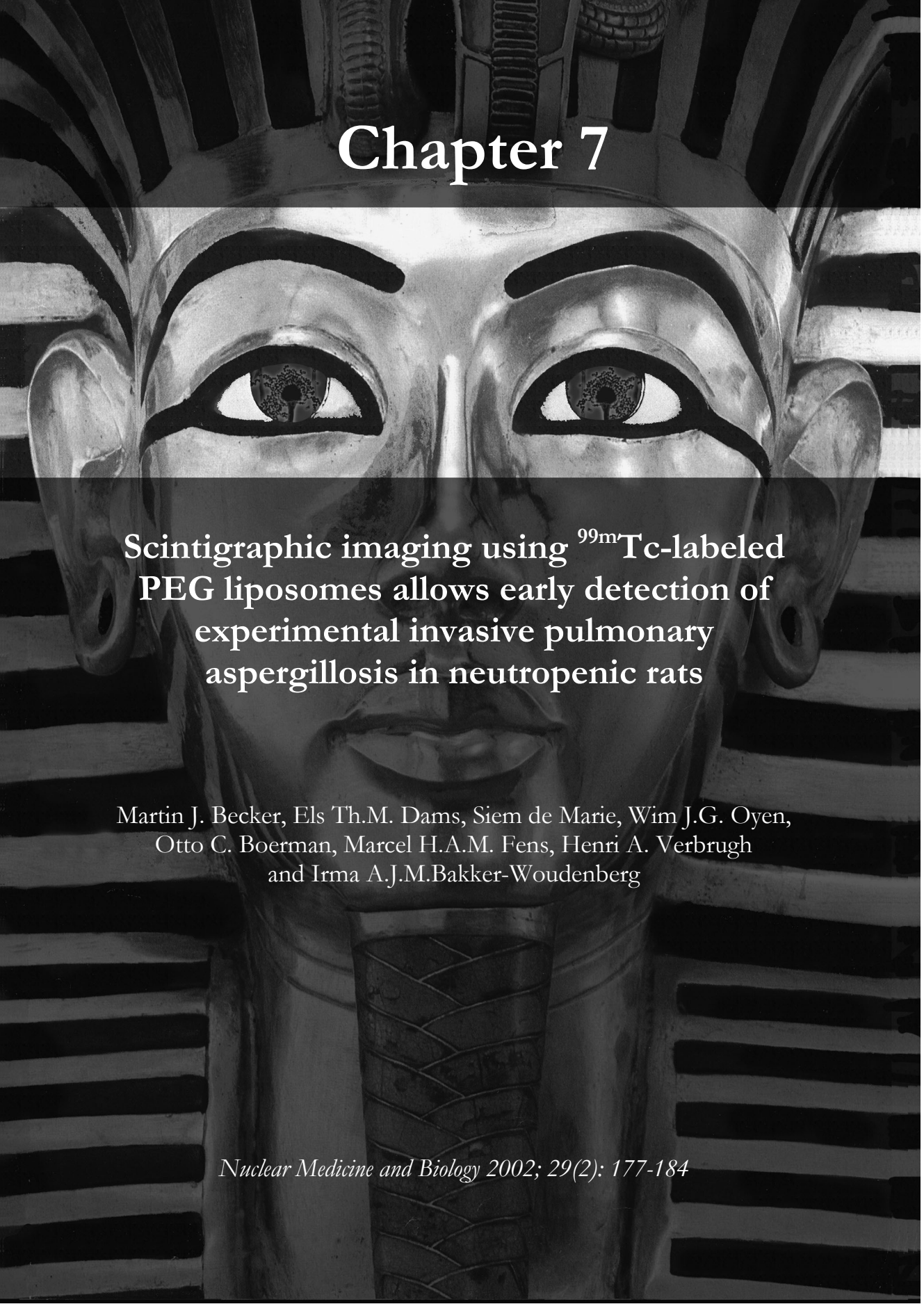

Chapter 7

Scintigraphic imaging using ^{99m}Tc-labeled PEG liposomes allows early detection of experimental invasive pulmonary aspergillosis in neutropenic rats

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ABSTRACT

The value of scintigraphic imaging using ^{99m}Tc-labelled poly(ethyleneglycol) (PEG) - liposomes for detecting invasive pulmonary aspergillosis at different stages of the disease was investigated in a rat model. At 24, 48, 72, 120 and 168 h after fungal inoculation scintigraphic images were obtained and biodistribution of the radiolabel was determined. Findings were compared with serum galactomannan detection and other parameters of progression of fungal infection.

At 48 h liposomal uptake in the infected left lung was increased significantly and 82% of the scintigraphic images was assessed positive. Serum galactomannan was only detected at 72 h and later. Liposomal uptake in the infected left lung increased over time and was significantly correlated with both the size of the pulmonary hemorrhagic lesion and the levels of circulating galactomannan. It was concluded that scintigraphic imaging using ^{99m}Tc-PEG-liposomes allows early detection of invasive pulmonary aspergillosis in this model, and that liposomal uptake in the infected lung was strongly associated with the severity of the disease.

INTRODUCTION

Invasive pulmonary aspergillosis (IPA) is a major cause of morbidity and mortality in neutropenic patients [1]. Early detection and diagnosis of the infection is of great importance, since it has been shown that early antifungal treatment significantly improves outcome [2,3]. In the diagnostic process of IPA, a positive CT scan is one of the first indicators for the disease. CT scanning is more sensitive than radiography and may reveal consolidations suggestive of fungal infection such as the "halo" and "air-crescent" signs [4]. Other steps in the diagnostic process are efforts to culture the fungus from sputum, broncho-alveolar lavage fluid (BALF) or material obtained by (transcutaneous) lung biopsies. IPA can be proven by providing histopathological evidence of mycelial growth in pulmonary tissue. However, none of these methods can reliably detect or diagnose IPA in its very early stage. New diagnostic tests are therefore under investigation. In this respect, the detection of circulating galactomannan has shown promising results [5].

Scintigraphic imaging techniques may have an additional value in the diagnostic process of IPA. In contrast to radiological imaging methods such as X- ray and CT, scintigraphic imaging does not depend on structural changes in the lung caused by the presence of the fungus, but on physio-chemical changes in the lung induced by the microorganism [6]. Also, this technique offers the possibility of a rapid whole-body evaluation, and therefore may be useful for the detection of extra-pulmonary disseminated fungal infection. At present, ^{67}Ga-citrate is the radiopharmaceutical of choice for the imaging of lung / thoracic infections in neutropenic hosts [7]. However, this imaging agent has unfavourable imaging characteristics and causes relatively high radiation exposure [8,9]. Several other scintigraphic agents are currently under investigation. ^{111}Indium-labeled non-specific human- immunoglobulin G (HIG) has shown good results in detecting infection in febrile granulocytopenic patients [10] . Other promising newly developed imaging agents are radioactively labelled long-circulating liposomes. Coating the liposomal surface with poly(ethyleneglycol) (PEG) avoids rapid recognition by the mononuclear phagocyte system and as a consequence results in prolonged residence time of the liposomes in the circulation which results in substantial liposomal localisation in infected tissue [11-13]. Increased capillary permeability with resulting liposomal extravasation is thought to be important for this localisation, but the exact mechanism by which PEG-liposomes localise is not yet well understood [14,15]. Liposomes can be labelled with ^{67}Ga, ^{111}In or ^{99m}Tc. Of these three labels ^{99m}Tc provides better image quality and lower radiation exposure compared to the other two agents [16].

In a previous study we found that IPA in neutropenic rats could be detected by scintigraphic imaging at 72 h after fungal inoculation [17]. In this study ^{99m}Tc-PEG-liposomes had scintigraphic imaging qualities that were superior to ^{67}Ga-citrate and slightly better than those of labelled polyclonal IgG (^{99m}Tc-HYNIC-IgG). In the present study scintigraphic imaging using ^{99m}Tc-PEG-liposomes

was performed in different stages of the fungal disease. The value of scintigraphic imaging for early detection of IPA was determined. In addition, the relation between imaging characteristics and parameters for progression and severity of infection was investigated.

MATERIALS AND METHODS

Infection model of IPA

The animal model used was based on the rat model of *Klebsiella pneumoniae* pneumonia described by Bakker-Woudenberg et al. [18], and adapted for *Aspergillus fumigatus* [19,20]. Some modifications were made to lengthen the survival time of the rats.

Specified pathogen free female RP strain albino rats (18-25 weeks old, 185-225 g) were used. Neutropenia was induced by cyclophosphamide (Sigma-Aldrich Chemie, Steinheim, Germany) 75 mg/kg i.p. five days before inoculation, followed by repeated doses of cyclophosphamide 60 mg/kg i.p. at 1 day before and 3 and 7 days after inoculation. This protocol resulted in granulocyte counts of less than 0.1×10^9 / L on the day of inoculation and afterwards. To prevent bacterial superinfections animals received ciprofloxacin (660 mg/L) and polymyxin E (100 mg/L) in their drinking water during the whole experiment. Starting one day before inoculation, daily i.m. amoxicillin (40 mg/kg/day) was added to this regimen for the remainder of the experiment. On the day of inoculation i.m. gentamìcin (6 mg/kg) was added. For infection of the rats a strain of *A. fumigatus* was used, that was originally isolated from an immunocompromised patient with IPA. Once every month the strain was passed into rats to maintain its virulence. The rats were anaesthetised with Hypnorm (Janssen, Belgium), 0.1 ml / rat i.m. together with Nembutal (Sanofi Sante, The Netherlands), 0.3 ml / rat i.p. of a 4 times diluted solution in distilled water). Under general anaesthesia the left main bronchus was intubated. A cannula was passed through the tube and the left lung was inoculated with 2×10^4 *A. fumigatus* conidia in 0.02 ml phosphate buffered saline (PBS, pH 7.4). This resulted in a one-sided IPA. Mortality rate was ± 50% on day 7, and 90-100% on day 12 after inoculation. In approximately half of the rats fungal dissemination to extra-pulmonary organs occurred, especially to the liver. Blood cultures for Aspergilli always remained negative. Rats that served as controls were inoculated with 0.02 ml PBS or non-viable conidia. Non-viable conidia were obtained by incubating conidia in 10% formaldehyde solution in PBS for 24 h and subsequent repeated washing with PBS. No viable conidia remained in this suspension, as checked by culture on Sabouraud- agar (Oxoid, Basingstoke, UK).

The experimental protocols adhered to the rules laid down in The Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The present protocols were approved by the Institutional Animal Care and Use Committee of the Erasmus University Rotterdam.

Radiopharmaceuticals

^{99m}Tc-PEG-liposomes were prepared as described previously [21] . The liposomes were composed of egg phosphatidylcholine (EPC), the poly(ethylene derivative of distearoylphosphatidyl-ethanolamine (PEG-DSPE), cholesterol and the hydrazino-nicotinamide derivative of distearoylphosphatidylethanolamine (HYNIC-DSPE) in a molar ratio of 1.85:0.15:1:0.07. The particle size distribution was determined by dynamic light scattering with a Malvern 2000 system equipped with a 25 mW Neon laser (Malvern Instruments Ltd, Malvern, UK). As a measure of particle size distribution of the dispersion, the polydispersity index was determined. This index ranges from 0.0 for an entirely monodisperse dispersion, up to 1.0 for a completely polydisperse dispersion. The mean diameter of the liposomes was 85 nm with a polydispersity index of 0.1. Preformed HYNIC-PEG-liposomes were labelled with ^{99m}Tc as described previously [21]. The radiochemical purity of the labelled PEG-liposomes was determined using instant thin-layer chromatography (ITLC) on ITLC-SG strips (Gelman Sciences, Inc., Ann Arbor, MI) with 0.15 M sodiumcitrate (pH 5.0) as the mobile phase and verified by elution on a PD-10 column. Labelling efficiency exceeded 95% and the ^{99m}Tc-PEG-liposomes were administered i.v. without any further purification.

Study design

At 0, 24, 48, 96, and 144 h after fungal inoculation, groups of rats were injected i.v. via the tail vein with 10 MBq ^{99m}Tc-PEG--liposomes (± 1 μmol phospholipid per rat). Imaging with this agent was optimal 24 h after injection [17] . At this time point the animals were anaesthetised with a mixture of halothane, nitrousoxide and oxygen, and were placed prone on a single head camera equipped with a parallel hole, low energy collimator. Images (100,000 counts per image) were obtained and stored in a 256 x 256 matrix. After acquiring the image, rats were killed with pentobarbital i.p. and dissection was performed. Blood was obtained by aortic puncture. Left and right lung, liver, kidneys and spleen were removed, weighed, and their radio-activity was measured in a shielded well-type gamma counter (Wizard, Pharmacia-LKB, Sweden). To correct for physical decay and to calculate uptake of the radiopharmaceuticals in each tissue sample as a fraction of the injected dose, aliquots of the injected dose were counted simultaneously. Lungs and liver were homogenised and cultured quantitatively on Sabouraud-agar.

Assessment of scintigraphic images

All scintigraphic images were evaluated by two observers (both medical specialists in nuclear medicine) who were unaware of the image being obtained from an infected or uninfected rat. Images were scored as either negative (no preferential accumulation of activity in the left lung region), positive (preferential accumulation of activity in the left lung region) or strongly positive (strong preferential accumulation accumulation of activity in the left lung region). When there was disagreement between the observers

they discussed the result of the scintigraphic image with each other until they reached the same conclusion.

Pulmonary macroscopic hemorrhagic lesion size

Macroscopic pulmonary pathology of the left lung was determined by measuring the size of the hemorrhagic lesion as percentage of the total lung surface. This was scored from photographs of the lungs taken immediately after dissection by measuring the lesion size on the photograph.

Histology

Separate groups of rats were sacrificed on indicated intervals after inoculation. The left and right lung were fixed with formalin and embedded in paraffin. Every lung was cut at 3 levels ± 1 mm apart. At every level 2 adjacent sections were obtained, of which one was stained with haematoxylin - eosin (H&E) and the other with Grocott's methenamine silver [22]. Lesions were graded based on the following five parameters: (1) hyphal growth, (2) leukocyte accumulation, (3) tissue invasion, (4) hemorrhagic infarction, (5) tissue necrosis. In each category a semi-quantitative score was assigned. Hyphal growth was scored as: only conidia visible (-), mostly small hyphae (< 20 μm) present (+), or mostly large hyphae (> 20 μm) present with the majority of the hyphae showing branching (++). Leukocyte accumulation was scored as: no infiltrate visible (-), few leukocytes or thin leukocytic rim around the fungal foci (+), or clear leukocytar infiltrate around the fungal foci (++). Tissue invasion was scored as : conidia or hyphae confined to bronchial lumen (-), invasion of bronchial wall (+), invasion of bronchial wall and angioinvasion (++), or extensive hyphal growth through tissue and extensive angioinvasion (+++). Hemorrhagic infarction was scored as: no haemorrhagia visible (-), hemorrhagic infarction visible only direct around fungal foci (+), or extensive hemorrhagic infarction throughout the lung tissue (++). Necrosis was scored as: no necrosis visible (-), necrosis only inside and directly around the fungal foci (+), or extensive tissue necrosis (++).

Fungal culture

From the rats the left and right lung, liver and kidneys were aseptically removed and disrupted in 20 ml phosphate buffered saline (PBS) using a tissue homogeniser for 45 sec at 10,000 rpm. Serial ten-fold dilutions were spread onto Sabouraud agar plates and incubated at 37 °C for 36 h, after which colony forming units (cfu) were counted.

Galactomannan detection

Concentrations of circulating galactomannan were measured as described before [23] Briefly, 300 μl of each serum sample was used in a sandwich ELISA (Platelia Aspergillus, Sanofi Diagnostics Pasteur). Each plate contained a calibration curve derived from rat serum samples containing 0, 1, 1.5, 2, 3, 4, 6, 8 and 12 ng/ml galactomannan. A test sample was considered positive when optical density at 450 nm

was higher than the cut-off sample (i.e. 1.0 ng/ml). The concentration of galactomannan in positive test samples was expressed as amount of nanograms galactomannan per ml serum.

Statistical methods
Linear regression was used to analyse the increase of left / right lung radioactivity ratio's over time. Associations between left / right lung ratios and assessment of scintigrams, pulmonary hemorrhagic lesion size, and circulating galactomannan levels were analysed by Pearson's correlation test.

RESULTS

Groups of neutropenic rats with left-sided IPA (n=7 to 18 per group) and uninfected controls (n=3 to 4 per group) were injected with ^{99m}Tc-PEG-liposomes at 0, 24, 48, 96 and 144 h after inoculation. One group of rats (n = 6) was inoculated with non-viable conidia and injected with labelled liposomes at 0 h only. 24 h after injection of liposomes (i.e. 24, 48, 72, 120 and 168 h after inoculation) a radioscintigram was obtained and biodistribution of the radiolabel and parameters of fungal disease were determined.

Biodistribution of ^{99m}Tc-PEG-liposomes
Uptake of the radiolabel was determined in the left lung, right lung, blood, spleen, liver and kidneys. The ratio of the activity per gram infected left lung tissue and gram uninfected right lung tissue was calculated to express liposomal uptake in infected vs. uninfected tissue. In uninfected rats, mean liposomal uptake in the left lung (% ID / g) was below 2 % at all time points (range 0.55-1.94%). Mean left / right lung ratio's in these rats were approximately 1 (range 0.76- 1.12) at all time points (data not shown). In contrast, in infected animals mean liposomal uptake in the left lung was above 2 % of ID /g at all time points, and a significant increase over time was found in left / right lung ratio's ($P < 0.001$) (Table 1). Mean left / right lung ratio's in infected animals were significantly elevated compared to uninfected animals at 48 h post inoculation ($P = 0.03$) and at all later time points ($P = 0.04$ to 0.0001) . The highest mean left / right lung ratio and mean % ID / g left lung was found at 168 h after inoculation. Also, at this time point liposomal uptake in the left lung was higher than in blood, as indicated by the left lung / blood ratio (1.94). Rats that were inoculated with non-viable conidia did not show increased left / right lung ratio's (Table 1). In the extra-pulmonary organs, the liposomes accumulated to a certain extent in the liver and spleen. Blood activity decreased over time, which was partly due to increased organ uptake and probably partly due to increased excretion of the tracer during the course of the disease.

Table 1. Biodistribution of ^{99m}Tc--PEG-liposomes in rats with invasive pulmonary aspergillosis at different stages of the disease.

Inoculation	Viable conidia					Non-viable conidia
Time after inoculation[1)]	24 h	48 h	72 h	120 h	168 h	24 h
N° of rats	8	11	7	18	15	6
Left lung (%ID/g)[2)]	2.47 ± 0.47	3.48 + 0.74	3.84 ± 1.03	2.42 + 0.32	4.94 ± 0.63	1.94 ± 0.20
Right lung (%ID/g)	1.75 ± 0.41	1.44 ± 0.23	1.37 ± 0.12	0.95 ± 0.07	0.96 ± 0.09	1.69 ± 0.15
Blood (%ID/g)	5.51 ± 0.16	4.11 ± 0.41	3.76 ± 0.34	3.01 ± 0.18	2.71 ± 0.18	5.55 ± 0.30
Spleen (%ID/g)	22.16 ± 1.87	24.59 ± 2.94	23.00 ± 2.74	20.45 ± 1.04	22.77 ± 2.08	19.73 ± 1.86
Liver (%ID/g)	2.09 ± 0.29	2.20 ± 0.20	2.29 ± 0.17	2.43 ± 0.15	3.06 ± 0.20	2.12 ± 0.11
Kidney (%ID/g)	1.47 ± 0.20	0.92 ± 0.09	0.91 ± 0.07	0.94 ± 0.04	1.03 ± 0.06	1.35 ± 0.20
Left/right lung ratio	1.61 ± 0.28	2.67 ± 0.63	2.83 ± 0.75	2.59 ± 0.27	5.91 ± 0.97	1.15 ± 0.11
Left lung/blood ratio	0.46 ± 0.08	0.83 ± 0.17	0.93 ± 0.20	0.85 ± 0.11	1.94 ± 0.26	0.40 ± 0.04

Data are shown as mean ± SEM.

[1)] time points are indicated at which scintigrams were obtained. ^{99m}Tc--PEG-liposomes were administered 24 h earlier.

[2)] percentage of injected dose per gram.

Table 2. Uptake of ^{99m}Tc- PEG- liposomes and parameters of fungal infection in rats with invasive pulmonary aspergillosis at different stages of the disease.

Time after inoculation[1)]	24 h	48 h	72 h	120 h	168 h
N° of rats	8	11	7	18	15
Liposomal uptake, left/ right lung ratio	1.61 ± 0.28	2.67 ± 0.63	2.83 ± 0.75	2.59 ± 0.27	5.91 ± 0.97
Scintigram assessment (% positive)	13% ± 13%	82% ± 12%	71% ± 20%	72% ± 11%	100% ± 0%
Scintigram assessment (% strongly positive)	0% ± 0%	0% ± 0%	14% ± 7%	11% ± 7%	67% ± 17%
Macroscopic lesion size (% of left lung surface)	0% ± 0%	3% ± 1%	15% ± 3%	25% ± 5%	48% ± 7%
Fungal culture of left lung (^{10}log no cfu)[2)]	3.98 ± 0.03	3.39 ± 0.07	3.67 ± 0.16	3.63 ± 0.08	3.69 ± 0.05
Galactomannan (% positive)	0% ± 0%	0% ± 0%	57% ± 20%	89% ± 8%	100% ± 0%
Galactomannan (concentration [ng/ml])	< 1.0	< 1.0	6.2 ± 2.2 [1)]	26.8 ± 12.0	74.8 ± 23.7

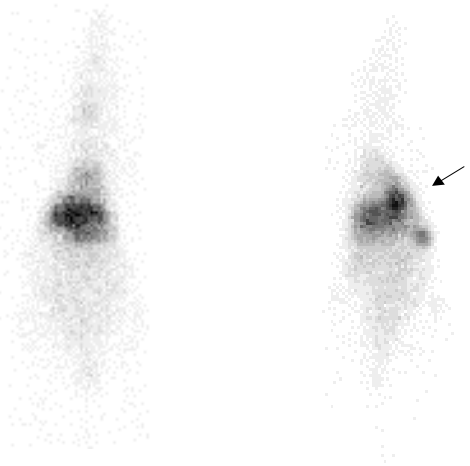
Data are shown as mean ± SEM

[1)] time points are indicated at which scintigrams were obtained. ^{99m}Tc--PEG-liposomes were administered 24 h earlier.

[2)] ^{10}log of number of colony forming units

^{99m}Tc liposomal pulmonary uptake and scintigraphic imaging results related to parameters of fungal infection

Representative scintigraphic images of rats with and without left-sided IPA are shown in Figure 1.



Uninfected 168h after fungal inoculaion

Figure 1. Scintigraphic images of rats with and without left-sided invasive pulmonary aspergillosis. Arrows indicate areas of elevated liposomal uptake in the left lung.

Assessment of scintigrams

Assessment scores of scintigrams and parameters of fungal infection progression are shown in Table 2. Of 17 uninfected controls, only positive for infection. In infected animals, the percentage of scintigrams that was assessed positive increased over time and was significantly correlated with the left / right lung ratio (P=0.003). At 24 h after inoculation a small percentage (13%) of the scintigrams were assessed positive. At 48 h after inoculation however, the majority of the scintigrams (82 %) were scored positive. Between 48 h and 120 h the total percentage of positive scintigrams was about constant, whereas the percentage of strongly positive scintigrams was higher at 72 h and 120 h. At 168 h, all scintigrams were assessed positive, with the majority (67%) being strongly positive.

Left lung pulmonary macroscopic lesion size

When the left lungs of infected animals were examined macroscopically, hemorrhagic lesions were seen that increased in size during the course of the disease. There was a highly significant correlation between the size of these hemorrhagic infarcts and the left / right lung ratio (P<0.0001, R=0.758) (Figure 2).

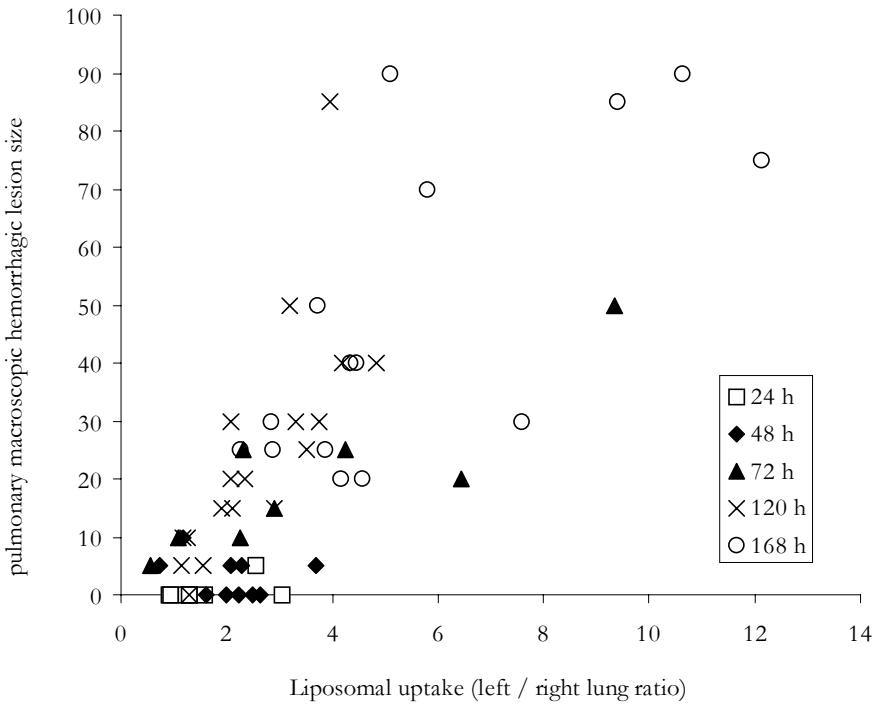


Figure 2. Correlation between size of pulmonary macroscopic hemorrhagic lesion and liposomal uptake, expressed as left / right lung ratio. Symbols mark the different time points after inoculation at which measurements were performed.

Fungal culture and disseminated fungal infection

Fungal culture of the left lung revealed highest number of cfu at 24 h after inoculation followed by an decrease at later time points. No association was found between number of cfu and progression of fungal disease or left / right lung ratio. Disseminated fungal infection from the left lung to the right lung was present in two out of seventeen rats at 120 h and one out of eight rats at 168 h, mean log cfu of *Aspergillus* being 1.78 ± 0.50. Disseminated fungal infection in the liver was present in one rat at 120 h and two rats at 168 h, mean log cfu being 2.03 ± 0.22. In none of the rats dissemination to both the right lung and the liver was found. The presence of disseminated fungal infection could not be observed on scintigrams. Also, the presence of disseminated fungal infection in either right lung or liver did not result in significantly increased uptake of ^{99m}Tc-PEG-liposomes in these organs. Mean liposomal uptake in the right lungs (% ID/g) of rats with disseminated infection in this organ was 1.08 ± 0.10, compared to 0.96 ± 0.07 in animals without disseminated infection. Mean uptake in livers (% ID/g)

was 2.99 ± 0.63 in rats with, and 2.43 ± 0.15 in rats without disseminated infection in this organ.

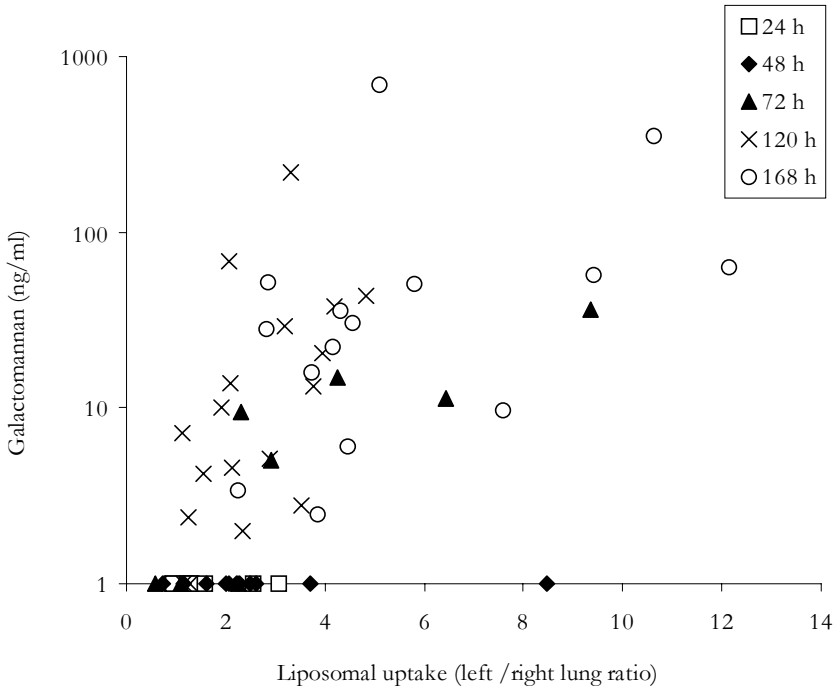


Figure 3. Correlation between concentration of galactomannan in serum and liposomal uptake expressed as left / right lung ratio. Symbols mark the different time points after inoculation at which measurements were performed.

Galactomannan detection

The proportion or rats positive for circulating galactomannan increased with progression of the disease. Compared to scintigraphic imaging, galactomannan detection was positive at a later stage in the course of the disease. At 48 h after inoculation 82% of the scintigraphic images was assessed positive and left / right lung ratio's in infected rats were elevated significantly compared to uninfected controls. At this time point in none of the rats circulating galactomannan could be detected. The first rats that were positive for galactomannan were found at 72 h after inoculation. From that time, mean levels of circulating galactomannan increased over time, and there was a significant association between galactomannan levels and left / right lung ratio ($P<0.0001$, $R= 0.8333$) (Figure 3). Overall, there was a significant correlation between positivity of scintigrams and galactomannan detection. Of all rats in which scintigrams were assessed positive, in 72% also galactomannan was detected, whereas of all rats in which scintigrams were assessed negative, in 27% galactomannan

was detected (P< 0.001). In addition, in all rats in which scintigrams were assessed strongly positive, galactomannan was detected .

Histopathology

In a separate experiment, 5 groups of 3 rats with IPA were sacrificed at 24 h, 48 h, 72 h, 120 h and 168 h after fungal inoculation, respectively. The lungs were removed and examined for histopathology. Results are shown in Table 3. Short hyphae were seen at 24 h after inoculation. Hyphae increased in length over time and showed radial growth and branching at later time points. Infiltrating leukocytes, predominantly granulocytes, were seen at all time points. The numbers of leukocytes were low, only seen as a thin granulocytic rim around the fungal foci, and there was no clear increase in amount of leukocytes over time. Tissue invasion was seen at 48 h and later, showing especially broncho-invasion at earlier stages, progressing to increasing angio-invasion at later stages. Small hemorrhagic infarcts were seen in some histological sections at 48 h, increasing over time to extensive tissue infarction at 168 h. Tissue necrosis was seen relatively late in the course of the disease, first observed at 72 h and increasing at later time points to significant tissue necrosis with widespread fungal growth.

Table 3. Histopathology of left lungs of rats with invasive pulmonary aspergillosis at different stages of the disease.

Time after inoculation	24 h	48 h	72 h	120 h	168 h
Hyphal growth	+	+/++	+/++	++	++
Leukocyte accumulation	+	+	+	+	+
Broncho and angio- invasion	-	+	+	++	++/+++
Hemorrhagic infarction	-	-/+	+	+/++	++/+++
Tissue necrosis	-	-	-/+	+	+/++

DISCUSSION

In the present study, we investigated whether scintigraphic imaging using ^{99m}Tc-PEG-liposomes could have an additional value in the diagnosis of IPA. In our rat model of left-sided IPA, we found that the majority of scintigraphic images (82%) were assessed positive already at 48 h after fungal inoculation. At that time liposomal uptake in the infected left lung was elevated significantly compared to the uninfected contralateral lung. Preferential accumulation of liposomes was associated with the presence of live fungal elements, since inoculation with saline only or killed conidia did not cause increased liposomal uptake. The mean left / right lung ratio in infected rats at 48 h was 2.67, and increased significantly over time until reaching a value of 5.9 at 168 h. Based on the findings of imaging of PCP using ^{111}In-IgG in two different studies, an infected tissue- to background ratio in rat lungs larger than 2 may predict successful imaging in patients [24,25].

We compared the results of scintigraphic imaging with a relatively new test for diagnosing IPA, the detection of circulating galactomannan. Favourable results using circulating galactomannan detection by sandwich ELISA were reported by Maertens et al. [5]. In their study of 71 patients with confirmed IPA, the sensitivity and specificity of this test was 92.6 and 95.4%, respectively. In more than half of the cases, antigenemia was detected before clinical suspicion of IPA (median, 6 days before), which indicates that antigenemia may occur early in the course of the fungal disease. In our rat model, at 48 h scintigraphy was positive in most rats, but circulating galactomannan could be detected only at 72 h and thereafter. Our rat model was characterised by an acute IPA, with around 50% of rats dying at day 7 after fungal inoculation. In humans the course of IPA generally extends over a longer period in time than in our animal model, therefore time differences may be larger and more time may be gained in early diagnosis of IPA.

Clinically, it may be especially interesting to investigate the value of scintigraphy neutropenic patients with fever, in which IPA is a major hazard. Currently, high-resolution computed tomography (HRCT)-scans are often used in these patients to evaluate the lungs after several days of fever refractory to antibiotics. Nevertheless, these CT scans may reveal negative or inconclusive results in the very early phase of IPA. Imaging using PEG- liposomes may have an additional value here, since CT-imaging depends on structural changes whereas scintigraphic imaging also depends on physio-chemical changes induced by the micro-organism. Also, the whole body can be evaluated at once by scintigraphy and therefore extrapulmonary localisations may be more easily located, whereas whole-body evaluations by CT are seldomely used. It must be noted however, that preferential localisation of PEG- liposomes at the site of infection is, as is also the case with CT-scan, not specific for invasive aspergillosis. In several other infection models, including a granulocytopenic rat model of bacterial pneumonia caused by *K. pneumoniae* in our institute [14], preferential localisation of PEG- liposomes has been described. Therefore, to diagnosediagnose IPA, a strategy in which scintigraphy is combined with more fungal-specific techniques such as galactomannan detection in serum and in fluid from site-directed broncho-alveolar lavage is necessary.

In our model, the uptake of ^{99m}Tc-PEG-liposomes increased over time, showing a clear correlation with the size of the hemorrhagic lesion ($P < 0.0001$). Also, in rats that were sacrificed at the same time point, we observed a relation between the macroscopic lesion size and the amount of labelled liposomes per gram lung. In addition, liposomal uptake was correlated with circulating galactomannan concentrations, which has been shown to be associated with severity of disease [23]. These results indicate that scintigraphy using radiolabelled PEG–liposomes may not only be useful for the early detection, but also for assessing the severity of disease. These findings are in agreement with data from our rat model of *K.pneumoniae* [11], showing a significant correlation between the amounts of localising PEG- liposomes in the infected left lung and the intensity of infection.

The mechanism by which PEG-liposomes accumulate in infected tissue is thought to be mediated by extravasation into inflammatory area as a result of locally enhanced vascular permeability [14,26,27], or by vascular leakage. In the early phase of infection in our study, liposomal uptake in the infected left lung was significantly elevated, while histopathological sections did not reveal hemorrhagic infarcts, and macroscopically no or very small hemorrhagic lesions were present. Therefore, it is likely that liposomal localisation at this time point was mainly due to increased vascular permeability. At later time points, hemorrhagic bleedings increased, as assessed macroscopically and by histological sections, indicating pulmonary bleeding also attributed to increase in liposomal localisation.

The involvement of leukocytes in liposomal extravasation was investigated by Schiffelers et al. in the model of *K. pneumoniae* pneumonia in rats [14]. They showed that liposomal localisation in the infected lung in leukopenic rats was similar to that in immunocompetent rats, demonstrating that the contribution of circulating leukocytes is limited. This explains why in our leukopenic rat model of invasive aspergillosis showing a minimal leukocytar infiltrate, a substantial localisation of liposomes was seen.

We conclude that in our model of persistently neutropenic rats scintigraphic imaging could be of value for the early detection of IPA, and that liposomal uptake correlates with severity of fungal disease. Although it must be noted that accumulation of liposomes in areas of inflammation is not specific for IPA, scintigraphy could be a useful tool in the diagnosis of human IPA.

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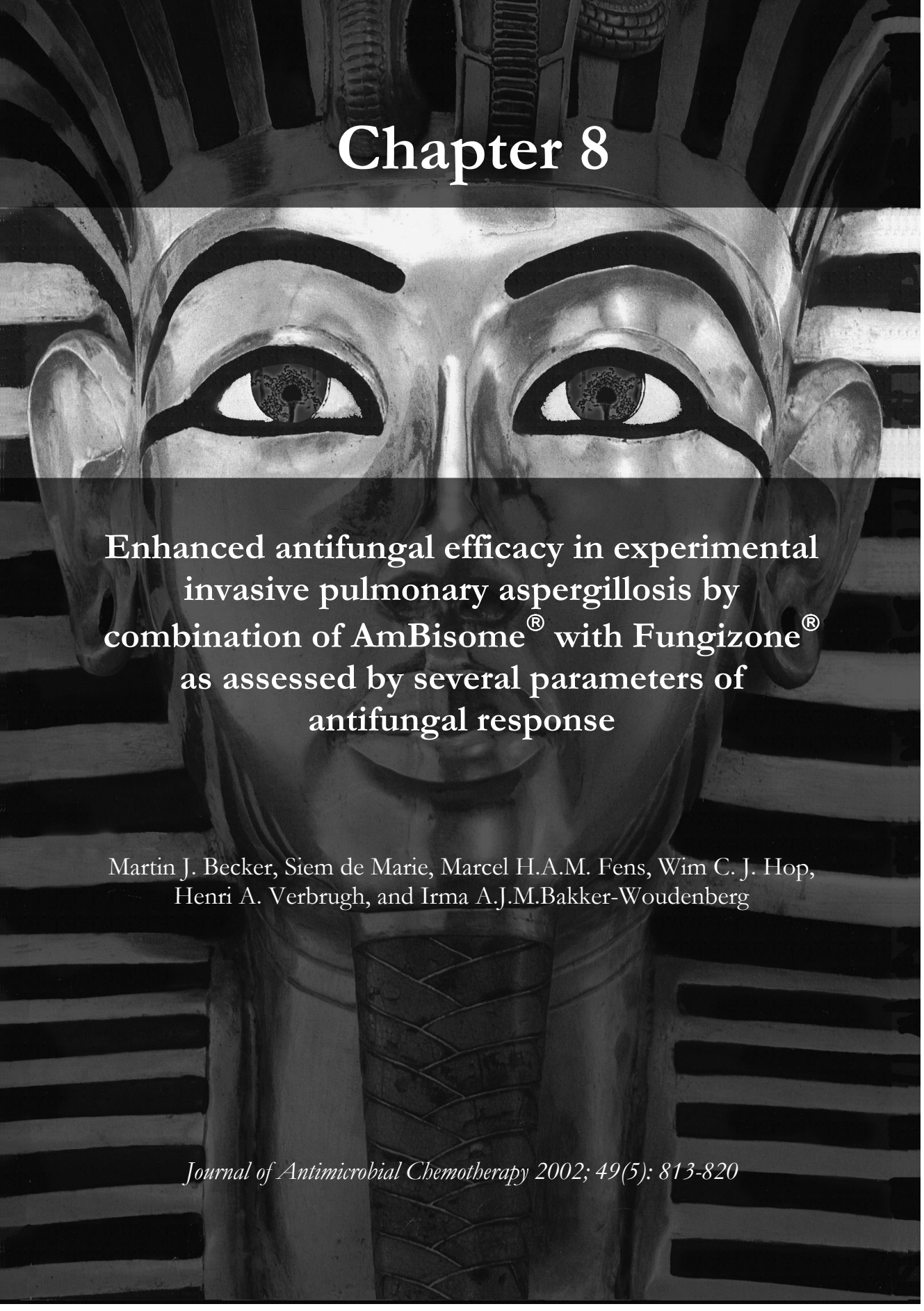

Chapter 8

Enhanced antifungal efficacy in experimental invasive pulmonary aspergillosis by combination of AmBisome® with Fungizone® as assessed by several parameters of antifungal response

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ABSTRACT

In common with a proportion of patients with invasive pulmonary aspergillosis, the efficacy of AmBisome® treatment regimens in our rat model remains suboptimal. To investigate whether this might be the result of initially low antifungal activity of amphotericin B at the site of infection when administered in the liposomal form, Fungizone® was added to AmBisome® at start of treatment.

Groups of granulocytopenic rats with left-sided invasive pulmonary aspergillosis received 10-day-treatment regimens with either AmBisome® 10 mg/kg/day (n=25) or AmBisome® 10 mg/kg/day combined with a single dose of Fungizone® 1 mg/kg at day 1 (n=27). Parameters of treatment response included survival, serum galactomannan, size and quality of pulmonary macroscopic lesions, lung weight, viable fungal counts (cfu) and chitin content of the infected lung, and extra-pulmonary disseminated fungal infection. In a separate experiment the significance of early start of treatment to obtain therapeutic efficacy was investigated.

Compared to untreated controls, both treatment regimens showed a significant increase in survival and change in parameters of fungal infection except left lung cfu. The combination treatment showed a significant increase in survival compared to AmBisome® monotherapy (P= 0.02) and a significant decrease in left lung chitin content (P=0.03). Differences in circulating galactomannan concentrations between the two treatment regimes approached significance (P=0.06). Delay in start of treatment from 16 h to 24 h after fungal inoculation resulted in a significant decrease in therapeutic efficacy (P=0.02).

It is concluded that efficacy of AmBisome® therapy can be enhanced by the addition of Fungizone® at the start of treatment. This is probably a result of active amphotericin B being immediately available in the lung at start of treatment.

INTRODUCTION

During the past decade, invasive aspergillosis has become an increasingly common opportunistic infection appearing primarily in patients receiving cancer chemotherapy or immunosuppressants [1]. Amphotericin B (Fungizone®) remains the first drug of choice in the treatment of patients with invasive pulmonary aspergillosis (IPA) [2]. However, treatment with Fungizone® is often unsuccessful [3,4] and its use is limited by its dose-related nephrotoxicity [5]. Several lipid-based formulations of amphotericin B have been developed to reduce the toxicity associated with conventional amphotericin B [6]. One of these lipid formulations is liposomal amphotericin B (AmBisome®). AmBisome® can be administered in higher dosages than Fungizone®, which may result in a better therapeutic index. However, although the efficacy of high-dose AmBisome® is equal to or better than Fungizone®, failure rates still give cause for concern [7,8]. One explanation for the limited success of AmBisome® in a proportion of patients might be a substantial reduction in the immediate bioavailability of free amphotericin B when the drug is liposome- encapsulated [9]. Clinical studies have shown that early start of treatment with amphotericin B is important for a successful outcome [10,11] therefore, low initial antifungal activity may cause a decrease in therapeutic efficacy. Evidence to support this hypothesis was found previously in our institution. Experimental studies showed that the addition of a single dose of Fungizone® at the start of a 10-day AmBisome®- regimen significantly increased survival of granulocytopenic mice with disseminated candidiasis [12].

In the present study, it was investigated whether the efficacy of AmBisome® in our model of IPA in persistently granulocytopenic rats could be improved by the addition of Fungizone® in the early phase of the disease. Several parameters for assessment of therapeutic response were used.

MATERIALS AND METHODS

Infection model of IPA

Our animal model, first described by Leenders et al. [13], was used with a few modifications. Specified pathogen free female RP strain albino rats (18-25 weeks old, 185-225 g) were employed. Profound granulocytopenia was induced by intraperitoneally (i.p.) administration of 75 mg/kg cyclophosphamide (Sigma-Aldrich Chemie, Steinheim, Germany) 5 days before inoculation, followed by repeated doses of 60 mg/kg 1 day before and 3 and 7 days after fungal inoculation. This protocol resulted in granulocyte counts of less than 0.1×10^9 / L on the day of fungal inoculation. To prevent bacterial superinfections, animals were given daily doses of 40 mg/kg amoxicillin intramuscularly (i.m.) starting 1 day before inoculation and a 6 mg/kg dose of gentamycin i.m. on the day of inoculation. In addition, rats received ciprofloxacin (660 mg/L) and polymyxin B (100 mg/L) in their drinking water throughout the

experiment. Rats were inoculated with a clinical isolate of *Aspergillus fumigatus* originally isolated from an immunocompromised patient with IPA. In accordance with NCCLS procedures [14], the MIC of amphotericin B for this strain was 0.4 μg/mL. Infection was established by intubation of the left main bronchus under general anaesthesia. A cannula was passed through the tube and the left lung was inoculated with 6×10^4 *A. fumigatus* conidia suspended in 20 μL of phosphate buffered saline (PBS).
Prior to experiments in infected rats, treatment regimes were administered to uninfected, granulocytopenic rats in which no noticeable toxicity was observed.
The experimental protocols adhered to the rules laid down in The Dutch Animal Experimentation Act (1977) and the published Guidelines on the Protection of Experimental Animals by the Council of the EC (1986). The present protocols were approved by the Institutional Animal Care and Use Committee of the Erasmus University Rotterdam.

Antifungal treatment
Antifungal agents were administered intravenously via the lateral tail vein to groups of rats daily. Treatment was started at 16 h after fungal inoculation, at which time hyphal growth was established. Fungizone® was obtained from Bristol-Meyers BV (Woerden, The Netherlands), and was diluted in 5% dextrose. AmBisome® was obtained from NeXstar pharmaceuticals (Wilrijk, Belgium) and diluted in 5 % dextrose. The following treatment regimens were investigated: 1) AmBisome® 10 mg/kg/day for 10 days; 2) AmBisome® 10 mg/kg/day for 10 days combined with a single dose of Fungizone® 1 mg/kg at day 1. When Fungizone® and AmBisome® were combined on the same day, AmBisome® was administered first and Fungizone within 20 minutes thereafter. Controls received no treatment, since previous studies showed that placebo treatment with either 5% dextrose or empty liposomes did not influence survival, levels of circulating galactomannan or other parameters of fungal infection (data not shown).

Parameters for efficacy of antifungal treatment

The survival rate of rats was monitored twice daily until day 11 after fungal inoculation. Circulating galactomannan (GM) was measured on days 3, 5, 7, 9 and 11 after fungal inoculation. At the end of the antifungal treatment rats had died or survived. Rats surviving on day 11 were sacrificed and dissected. In rats that died and sacrificed rats, the size of the pulmonary lesion of the left lung was measured, left lung weight was determined, organs were cultured for fungi and chitin assays were performed on the left lung homogenates.

GM detection
Blood for GM detection was sampled by puncture of the orbital venous plexus in the lateral canthus of the orbita. When cyclophosphamide, antifungal agents or antibiotics were administered to the rats on the same day as GM detection was performed, blood

samples for GM detection were taken within 2 hours before injection of these agents. Concentrations of circulating GM were measured as described previously [15]. Briefly, 300 μL of each serum sample was used in a sandwich ELISA (Platelia Aspergillus, Sanofi Diagnostics Pasteur, Belgium). Each plate contained a calibration curve derived from rat serum samples containing 0, 1, 1.5, 2, 3, 4, 6, 8 and 12 ng/mL GM.

Pulmonary macroscopic lesions and left lung weight

Pulmonary macroscopic lesions were observed in both untreated rats and treated rats. Angio-invasive lesions, seen as macroscopic dark-red lesions, were histologicaly characterised by extensive hyphal broncho- and angio-invasion and haemorrhagic infarction. Responsive lesions, seen as macroscopic light-red coloured lesions were histologicaly characterised by the presence of relatively short hyphae and little angio- and bronchio-invasion with resulting less hemorrhagic infarction. The size of the two types of pulmonary lesions was expressed as percentage of the total lung surface. The pulmonary lesion size was measured from photographs of the anterior of the lungs taken immediately after dissection.

Fungal cultures of organs

Left and right lungs were disrupted in 12 mL, livers in 20 mL phosphate buffered saline (PBS) using a tissue homogeniser (The Virtis Co. Inc., Gardiner, NY, USA) for 45 sec at 10,000 rpm. Colony forming units (cfu) in right lungs and livers were counted in 1:10 and undiluted homogenates. cfu in left lungs were counted in 1:10 and 1:100 dilutions, and the remaining 10.68 mL of homogenate was used for chitin assay. Dilutions of homogenates were spread onto Sabouraud agar plates. Undiluted homogenates were cultured according to the pour plate method. After incubation at 37 °C for 36 h the cfu were counted.

Chitin assay

A 10.68 mL aliquot of left lung homogenate was used for chitin assay, as described by Lehmann et al. [16]. In brief, the homogenate was centrifuged, resuspended in 4 mL of 3% sodium lauryl sulfate (SDS, Sigma chemical co., St Louis, USA) and heated at 100 °C for 15 min. After cooling, the pellet was washed once with distilled water, resuspended in 3 mL of 120% KOH solution and heated to 130 °C for 1h. Subsequently, 8 mL of ice-cold 75% ethanol was added, tubes were kept at 4 °C for 15 min and 0.3 mL of Celite suspension (Celite 545; Sigma) was added. After centrifugation, the pellet was washed with cold ethanol (40%) and cold distilled water respectively and suspended in 0.5 mL of $NaNO_2$ (5%) and 0.5 mL of $KHSO_4$ (95%). After centrifugation, volumes of the supernatant were mixed with 12.5% $NH_4SO_3NH_2$ followed by MBTH (3-methyl-benzo-2-thiazolone hydrazone HCl monohydrate; Sigma). After heating for 3 min, the supernatants were cooled, $FeCl_3$ $6H_2O$ (0.83%) was added and were allowed to stand for 30 min. The optical density at 650 nm was read in a spectrophotometer. The chitin content of the organs was expressed as

micrograms of glucosamine per organ. Final measurements of chitin were corrected for the loss of volume of homogenate.

Toxicity

To check for the presence of toxicity following antifungal treatment, renal and hepatic functions were monitored in treated rats. Renal function was measured by serum creatinine and blood urea antigen (BUN) after ten days of antifungal treatment. Hepatic function was measured by serum aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT).

Influence of treatment start-time on survival

In a separate experiment the influence of start time of treatment on therapeutic efficacy was investigated. In this experiment, treatment was started at 16 h after fungal inoculation in one group, and at 24 h in the other and survival of the rats was compared. All rats received the combination therapy.

Statistical methods

Differences in survival were assessed by the log rank test. Differences in proportions of animals with dissemination to the right lung and liver were examined by Fisher's exact test. Differences in parameters of fungal infection were assessed by student's T test with exception of differences in chitin amounts, which were calculated using Mann Witney's test.

To analyse circulating GM concentrations, logarithmically transformed GM values were taken, showing an approximate normal distribution. The mean increase of ^{10}log GM concentration with time was determined for each group of rats using repeated measurements ANOVA (random coefficients regression model) using the SAS Proc Mixed statistical computer package. The correlation between the measured concentrations of GM and mortality was assessed using Cox regression with ^{10}log GM concentration as the time-dependent variable.

RESULTS

Survival rate

Ninety-six percent of untreated rats died < 11 days after fungal inoculation. Both treatment regimens significantly improved survival compared to the untreated controls ($P < 0.0001$ for both treatment regimens), as shown in Figure 1. The addition of a single dose of Fungizone® 1 mg/kg at the start of treatment (day 1) to the 10-day AmBisome® regimen significantly improved survival compared to AmBisome® monotherapy ($P = 0.02$).

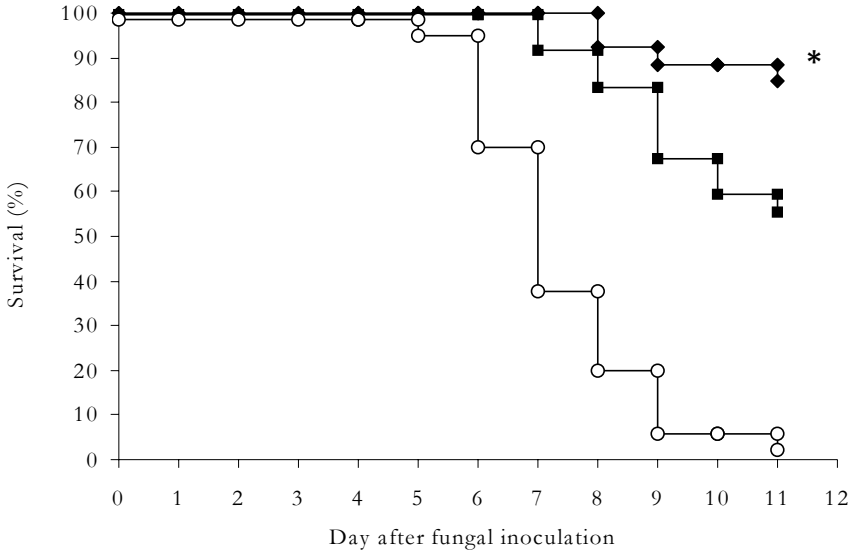

Figure 1. Survival of rats with IPA during antifungal treatment. Treatment was started 16 h after fungal inoculation and continued for 10 days. Diamonds, AmBisome® 10 mg/kg/day + Fungizone® 1 mg/kg on day 1; squares, AmBisome® 10 mg/kg/day; circles, untreated controls.* P = 0.02 compared with AmBisome monotherapy.

Concentrations of circulating GM

In both the untreated group and the treated groups, concentrations of circulating GM increased consistently over time (Table 1). The mean daily increases in ^{10} log GM concentrations for controls, AmBisome® monotherapy and combination therapy were 0.33 (± 0.02 SEM), 0.12 (± 0.02) and 0.08 (± 0.01) respectively. The mean increases in GM for the two treatment regimens were significantly lower than in controls ($P < 0.001$ for both regimens). The difference in ^{10} log GM increase between AmBisome® monotherapy and combination therapy approached significance ($P = 0.06$).

Using Cox regression, it was found that there was a significant correlation between GM concentration and mortality ($P < 0.001$). A ten-fold increase in GM concentration was associated with an approximate 6 – fold increase in mortality (relative death rate: 5.6; 95 % CI: 3.0-10.3). This correlation between GM concentration and mortality was not significantly influenced by the type of treatment administered ($P = 0.12$).

Table 1. Galactomannan serum concentrations in rats with IPA during different antifungal treatment regimens†

Day[1)]	Untreated controls		AmBisome® 10 mg/kg/day		AmBisome®10 mg/kg/day + Fungizone® 1 mg/kg on day 1	
	N° of rats [2)]	^{10}log GM conc	N° of rats	^{10}log GM conc	N° of rats	^{10}log GM conc
Day 3	20	0.83 ± 0.10	20	0.32 ± 0.12	17	0.26 ± 0.06
Day 5	19	1.42 ± 0.12	20	0.67 ± 0.14	17	0.51 ± 0.12
Day 7	11	1.95 ± 0.12	19	1.00 ± 0.11	16	0.85 ± 0.14
Day 9	5	2.55 +0.22	16	0.87 ± 0.17	16	0.69 ± 0.12
Day 11	1	2.65	13	0.78 ± 0.12	16	0.93 ± 0.14

Data are shown as mean ± SEM.

† Treatment was started 16 h after fungal inoculation and continued until day 10.

[1)] Day after fungal inoculation.

[2)] Galactomannan measurements were performed on all rats that survived at the indicated days after fungal inoculation.

Table 2. Parameters of fungal infection in rats with IPA receiving different antifungal treatment regimens

	Untreated controls	AmBisome® 10 mg/kg/day	AmBisome®10 mg/kg/day + Fungizone®1 mg/kg on day 1
N° of rats [1)]	28	25	26
Angio-invasive lesion left lung (%)	78 ± 5.8	27 ± 6.7*	17.6 ± 6.3*
Responsive lesion left lung (%)	5.7 ± 2.7	47 ± 7.2*	50 ± 7.7*
Weight left lung (g)	1.16 ± 0.07	0.74 ± 0.05*	0.73 ± 0.05*
^{10}log cfu in left lung	3.06 ± 0.08	3.05 ± 0.09	2.80 ± 0.07* ■
Culture positive right lung (%)	79 ± 8	24 ± 9*	12 ± 6*
^{10}log cfu in right lung	1.62 ± 0.19	0.47 ± 0.18*	0.19 ± 0.12*
Culture positive liver (%)	75 ± 8	0 ± 0.0*	0 ± 0.0*
^{10}log cfu in liver	1.01 ±0.09	0 ± 0.0*	0 ± 0.0*
Left lung chitin content [2)] (μg glucosamine)	15.4 (0-36.0)	4.7 (0-42.7)*	0.5 (0-57.5)* ■

Data are shown as mean or percentage ± SEM, except [2)] chitin contents, which are shown as median (range).

[1)] Measurements were performed on rats that died during treatment and on rats that were sacrificed at the end of treatment.

* $P < 0.05$ compared to untreated controls

■ $P < 0.05$ compared to rats receiving AmBisome® monotherapy

Pulmonary macroscopic lesions and left lung weight

Antifungal treatment had an effect on the weight and macroscopic appearance of the left lung (Table 2). A shift was observed from angio-invasive lesions in untreated animals to responsive lesions in both treatment groups. Histologicaly, angio-invasive lesions showed extensive fungal broncho- and angio-invasion and tissue hemorrhagia, all of which in responsive lesions were clearly reduced. Compared to controls, left lung weight was significantly lower in both treatment groups ($P < 0.001$ for both regimens). Compared to the AmBisome® monotherapy group, the number of angio-invasive lesions was lower in the combination therapy group, but this difference was not significant ($P = 0.31$).

Fungal cultures of organs

AmBisome® monotherapy did not reduce cfu cultured from the left lung (Table 2). In contrast, the combination therapy significantly reduced the cfu in the left lung compared to both the untreated controls ($P < 0.001$) and AmBisome® monotherapy group ($P = 0.04$). Both treatment regimens completely prevented dissemination of the liver.

Chitin content of the infected lung

Both the AmBisome® monotherapy and the combination therapy reduced chitin content in the left lung compared to untreated controls ($P = 0.03$ and $P < 0.01$ respectively). Compared to AmBisome® monotherapy, the combination therapy significantly reduced left lung chitin content ($P = 0.03$).

Toxicity

There were no significant differences between the treatment groups in serum creatinine, BUN, ALAT and ASAT concentrations after 10 days of treatment (data not shown).

Influence of treatment start-time on survival

In a separate experiment, it was investigated whether delay of start of treatment would have an effect on the therapeutic efficacy in our model. We compared the efficacy of the combination therapy when started at 16 h ($n = 10$), or at 24 h after fungal inoculation ($n = 12$) (Figure 2). A significant reduction in survival was seen when start of treatment was delayed from 16 h to 24 h ($P=0.02$)

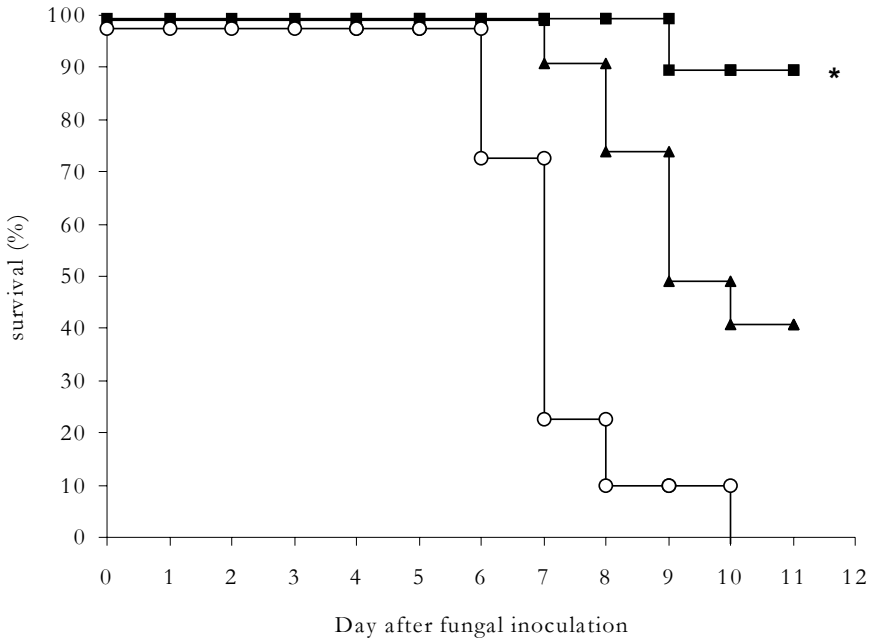

Figure 2. Survival of rats with IPA during antifungal treatment with AmBisome® 10 mg/kg/day plus Fungizone® 1 mg/kg on day 1. Treatment was started 16 h after fungal inoculation in one group of rats and at 24 h in the other. Squares, start of treatment at 16 h; triangles, start of treatment at 24 h; circles, untreated controls. * P = 0.02 compared with rats in which therapy was started at 24 h.

Correlation between parameters of fungal infection and survival in treated rats

To investigate which parameters of fungal infection were associated with survival in rats receiving antifungal treatment (either AmBisome® monotherapy or combination therapy), we compared the results of rats that died during treatment with those of rats that survived until the end of treatment (Table 3). In the surviving animals the size of the responsive lesion was significantly larger, while the angio-invasive lesion was smaller. In addition, the parameters of fungal load were lower in surviving animals with the left lungs containing significantly less cfu and chitin compared to animals that died. The number of cfu in the right lungs was also lower in surviving rats and the difference in the percentage of rats with dissemination approached significance.

Table 3. Comparison of the parameters of fungal infection in rats that died during treatment† with rats that were sacrificed at the end of treatment

	Treated rats that died before end of treatment	Treated rats that survived until end of treatment	*P* value
N° of rats	15	35	
Angio-invasive lesion left lung(%)	41 ± 9	14 ± 5	0.008
Responsive lesion left lung (%)	15 ± 5	35 ± 6	< 0.001
Weight left lung (g)	0.74 ± 0.06	0.74 ± 0.04	0.982
^{10} log cfu in left lung	3.1 ± 0.09	2.83 ± 0.07	0.02
Culture positive right lung (%)	33 ± 12	11 ± 5	0.06
^{10} log cfu in right lung	0.66 ± 0.25	0.18 ± 0.09	0.04
Left lung chitin content[1)] (μg glucosamine)	20.4 (0-42.7)	5.1 (0-57.5)	0.004

Data are shown as mean or percentage ± SEM, except [1)]chitin contents which is shown as median (range).
† Treatment was started 16 h after fungal inoculation and continued until day 10.

DISCUSSION

In this study, we compared the therapeutic efficacy of two treatment schedules using a clinically relevant animal model of invasive pulmonary aspergillosis (IPA) during persistent neutropenia. In this model, Leenders et al. showed that a 10-day treatment with high doses (10 mg/kg) of AmBisome® did not result in increased survival compared to a 10-day treatment with Fungizone® 1 mg/kg [13]. Clinical studies in neutropenic patients have also shown that AmBisome® treatment is not always superior to standard Fungizone® therapy [7,8,17].

The aim of our study was to investigate whether the efficacy of high doses of AmBisome® could be enhanced by increasing the immediate availability of the biologically active drug in the early phase of treatment. This hypothesis was based on studies performed in our laboratory by van Etten et al. in a mouse model of invasive candidiasis [9,12,18]. In biodistribution studies that measured the levels of amphotericin B in the lung at various time points, after a single injection of 0.3 mg/kg Fungizone® they found that amphotericin B could be detected in the lung at all time points from 5 min to 12 h. In contrast, after a single injection with 7 mg/kg AmBisome®, concentrations of amphotericin B in the lung could not be detected until 12 h and later. In addition, they reported that the *in vitro* antifungal activity of AmBisome® during short-term exposure (6 h) of *Candida albicans* was significantly less than that of Fungizone®. The latter finding was explained by a relatively slow release of amphotericin B from the liposomes. Furthermore, they found that addition of a single

dose of Fungizone® to a 5-day regimen of AmBisome® at the start of treatment significantly increased survival and decreased fungal load in the kidney of granulocytopenic mice with disseminated candidiasis.

Our findings are in accordance with these studies. In our rat model of IPA we have also observed that rat survival increased significantly when a single dose of Fungizone® was initially added to a 10-day AmBisome® regimen. When higher doses of AmBisome® (up to 30 mg/kg) were administered on day one and 10 mg/kg AmBisome® on day 2 to 10, no improvement compared to AmBisome® monotherapy was seen (data not shown), indicating that changing the absolute amount of AmBisome® on day one had no significant impact. Multiple doses of Fungizone® 1 mg/kg (at day 1,3 and 5, or at day 1 and 6, or twice at day 1 combined with the 10-day AmBisome® regimen did not further enhance survival compared to a single addition of Fungizone® 1 mg/kg at day 1 (data not shown). This indicates that only addition of Fungizone® at day one is relevant for improving survival. In addition, an early antifungal activity was shown to be a highly important factor since an 8-hour delay in starting antifungal treatment resulted in significant reduction in survival. Therefore, it is likely that the improved antifungal efficacy by the addition of Fungizone® to the AmBisome® regimen at day 1 is caused by increased bioavailability of amphotericin B in the lung in the early phase of the disease.

In addition to survival, we investigated the value of several parameters of fungal infection as markers for treatment response. Antifungal treatment appeared to have an effect on most of these parameters. Concentrations of serially sampled serum GM were significantly lower in both treatment groups compared to untreated controls while the difference between treatment groups approached significance. Other investigators have also described a suppressive effect of antifungal treatment on circulating GM concentrations in both animal models and patients with IPA [19,20]. In addition, increases in GM concentrations in our model were associated with a significantly increased mortality. This indicates that serum GM concentrations are a relevant parameter for measuring treatment response.

Antifungal treatment resulted in a reduction in lung weight and size of the dark-red coloured haemorrhagic angio-invasive lesion and an increase of the size of the light-red coloured responsive lesion. The size of this responsive lesion was also associated with increased survival, as opposed to the angio-invasive lesion. Others have also described decreased haemorrhagic lesions in a rabbit model of IPA and increased lighter coloured "resolving" lesions under antifungal treatment [21]. These findings suggest that these lesions reflect an important aspect of the pathogenesis of IPA. Since responsive lesions represented areas with reduced broncho-angio invasion and hemorrhagia, inhibition of the invasive spread of the fungus is probably important for successful antifungal treatment.

The numbers of cfu cultured from the left lungs of rats receiving combination therapy were lower than those of rats receiving AmBisome® monotherapy. However, compared to untreated controls the cfu of the AmBisome® monotherapy group were not

significantly decreased. This may be explained by the fact that the number of cfu does not represent the real viable fungal load: larger hyphae represent a larger fungal load but not necessarily a larger number of cfu [22]. In accordance with this, several studies reported that the numbers of cfu cultured from the organs in their animal models of IPA did not significantly increase over time [16,23,24], in contrast to quantitatively measured fungal DNA in one of these studies [24]. Chitin may be a more reliable indicator for fungal load than cfu, since it is a constituent from the fungal cell wall and the amount of chitin increases with the growth of the hyphae [16]. Indeed, we found a significant decrease in chitin amount in the left lung in both treatment groups compared to untreated rats, as well as significant differences between the two treatment groups.

In conclusion, we have demonstrated that the addition of a single dose of Fungizone® at the start of AmBisome® treatment enhanced therapeutic efficacy of AmBisome® monotherapy. Apart from survival, parameters of fungal disease that best represented increased treatment response were left lung chitin content and serum GM concentrations.

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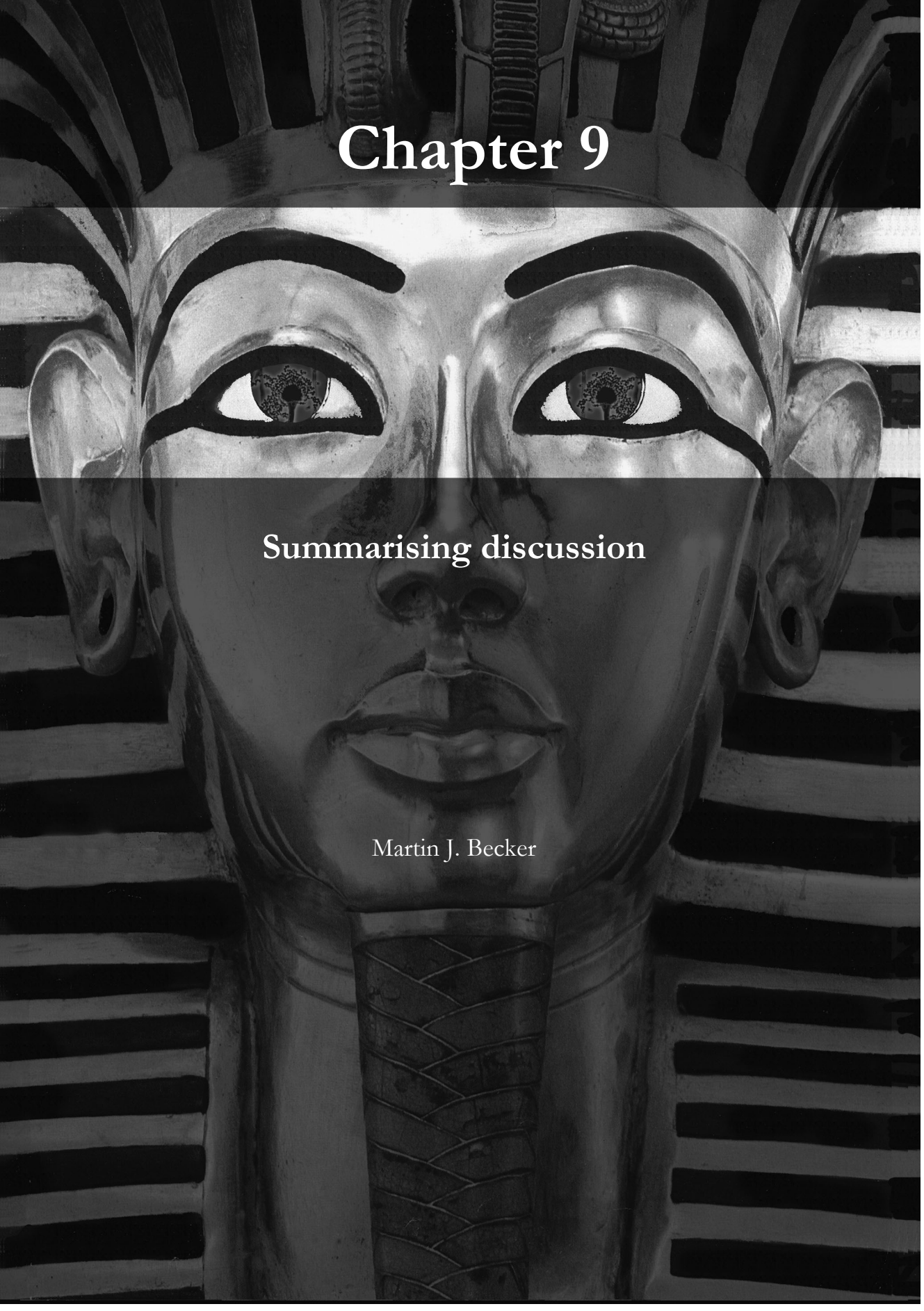


Chapter 9

Summarising discussion

Martin J. Becker

SUMMARISING DISCUSSION

Invasive pulmonary aspergillosis (IPA) is still a major problem among severely immuno-compromised patients, especially haemato-oncological patients receiving chemotherapy. The reasons for this are the following:

- There is still no reliable tool to diagnose the disease with high sensitivity and specificity in an early phase of the disease.
- There is no treatment regime that results in sufficiently high cure rates, and therefore the mortality and morbidity of the disease are still unacceptable high.

Numerous studies performed in an effort to improve the therapeutic outcome of patients with IPA have been published. However, several problems are encountered when investigating IPA:

- Clinical studies on IPA are difficult to perform because histologically proven cases are rare and patient groups can be heterogeneous. Therefore, it is difficult to test and compare new diagnostic – and treatment regimes.
- Adequate animal models of IPA are rare, and models vary widely and are often poorly defined.
- Little is known about the exact pathogenesis of IPA. Improved insight into the pathogenesis may lead to new diagnostic- and treatment strategies.

The present thesis addresses the above-mentioned problems as follows:

- The pathogenesis and host response in IPA were investigated in a rat model in order to gain more insight into the mechanisms of the course of the disease, and to obtain a better definition of our animal model.
- New tools for diagnosing IPA, i.e. galactomannan (GM) detection, PCR and scintigraphy were evaluated in the rat model.
- GM detection in serum and broncho-alveolar lavage (BAL)-fluid was evaluated haemato-oncological patients receiving chemotherapy.
- Pulmonary lesions, number of colony forming units (cfu), GM concentrations and chitin amounts were evaluated for monitoring IPA in the rat model.
- New treatment regimes were investigated in the animal model using a new antifungal drug formulation, i.e. liposomal amphotericin B (L-AMB).

PATHOLOGY, PATHOPHYSIOLOGY AND HOST RESPONSE

Pathology

Macroscopic and microscopic pathology were investigated in our rat model in **Chapters 3, 6 en 7**. Macroscopically, dark-red pulmonary infarcts, increasing in size over time, were seen. Microscopically, broncho-and angio-invasion was seen, necrotic lesions with radial growth of hyphae, surrounded by a leukocytic rim and tissue haemorrhagia. These findings indicate that histo-pathology in our model was

comparable to patients with IPA, and serve as an additional argument that the rat model is suited to study the pathophysiology of IPA.

Pathophysiology

Very little is known about the pathophysiology of IPA in patients, whereas more insight into the disease may help develop new diagnostic and / or treatment strategies. In addition, more insight into the pathophysiology of IPA in our rat model will help define our model better. For example, wether the cause of death of our rats with IPA is due to respiratory or circulating failure was unclear. **Chapter 2** describes the pathophysiology of IPA in our animal model, with emphasis on pulmonary function. Rats with IPA showed a decrease over time in body temperature and in body weight. An increase in respiratory distress was seen over time, suggesting severe pulmonary dysfunction. Compliance and lung-surfactant function were significantly decreased in the infected left lungs of rats with IPA. However, O_2 and CO_2 levels in arterial blood were not significantly different between infected rats and uninfected controls. Probably, the uninfected right lung compensated for the dysfunction of the infected left lung until a late stage in the disease.

Arterial blood pressure was significantly decreased in infected rats compared to uninfected rats. Thus, circulatory failure was a major feature in the terminal phase of IPA in our model, and it is likely that this plays a more important role in causing death of the rats than pulmonary dysfunction. Whether the same is true for patients with IPA is not reported in the literature and is an interesting subject of investigation.

Host response

In the pathogenesis of severe bacterial infections, cytokines and chemokines play an important role. Septic shock for example, is thought to be mediated by pro-inflammatory cytokines, especially TNF-α. There is evidence that cytokines and chemokines also play an important role in invasive aspergillosis. Studies showed that resistance to systemic invasive aspergillosis was associated with increased production of Th1-associated cytokines, whereas Th2-associated cytokines were related with disease progression. In **Chapter 3**, we investigated the pro-inflammatory cytokines TNF-α and IL-1β, the Th1- associated cytokine IFN-γ, the Th2 associated cytokines IL-4, IL-6 and IL-10 and the chemokines MIP-2 and MCP-1 in lungs and serum of rats with IPA. None of the pro-inflammatory cytokines was elevated in rats with IPA. This is a remarkable observation, since this is substantially different compared to severe bacterial infections. As described in **Chapter 2**, in rats with severe IPA circulatory failure occurred. Apparently, this circulatory failure develops independent of TNF-α and IL-1. Possibly, other factors may play a role in IPA, such as a direct effect of fungal toxins on the host. Of the other cytokines, only IL-6 was elevated in the infected left lung. This indicated a Th2 response in our model, which is in agreement with other studies in models of invasive aspergillosis.

The chemokines MCP-1 and MIP-2 were also elevated in the left lungs of rats with IPA. These substances have a function in attracting macrophages and neutrophils, which form the first and second line of the immune-defence against *Aspergillus*. Actually, in our study granulocytes were seen in microscopic sections of the infected lungs, around fungal foci (**Chapter 7**). This suggests that also in the neutropenic host these chemokines have a role in the defense against *A. fumigatus,* and are produced by the host in an attempt to attract macrophages and neutrophils.

DIAGNOSIS, MONITORING AND SCINTIGRAPHIC IMAGING

Diagnosis

Two new techniques for the early diagnosis of IPA have especially drawn attention the last few years: PCR and GM detection. Both tests have shown promising results in clinical settings. We first evaluated these tests in our animal model (**Chapter 4**). The advantage of using the model is that it is certain whether the animals have the disease or not, in contrast to patients. In addition, the exact time point of onset of the disease is known, allowing comparison of the tests in an early phase of the disease, which is relevant with respect to diagnosis. Compared to PCR, GM detection in blood /serum was earlier positive, and showed higher sensitivity. At day 7 after fungal inoculation, all rats were positive for GM, whereas only 41% of rats were positive for PCR. Also in BAL-fluid, GM detection was more often positive than PCR, especially in the later phase of the disease. In addition, GM detection in BAL-fluid was associated with hyphal growth of the fungus, whereas PCR was more associated with fungal colonisation. In conclusion, in the animal model, GM detection was an appropriate tool for the early diagnosis of IPA. This prompted us to evaluate this tool in patients at risk for IPA, which is described in **Chapter 5**. A clinical study was performed in neutropenic patients in three haemato-oncological wards. The GM test was applied in serum samples and computed tomography (CT)-based BAL-fluid samples. Despite serial sampling, the results for GM detection in serum were somewhat disappointing: sensitivity 47%, specificity 93%, positive predictive value (PPV) 73% and negative predictive value (NPV) 82%. In contrast, the results for GM detection in CT-guided BAL-fluid were much better: sensitivity 90%, specificity and PPV 100% and NPV 93%. In conclusion, in patients GM detection in BAL-fluid was clearly more useful than GM detection in serum. As a result, GM detection in BAL-fluid is now routinely used in our hospital in patients undergoing chemotherapy with a suspicion of IPA.

With increasing reports in the literature the results of GM detection in serum appears to vary widely from one centre to the other. The explanation for these inconsistent results might be differences between centres in patient populations, in antifungal treatment strategies or in antifungal prophylactic regimes. However, these are only speculative conclusions.

The role of the PCR in diagnosing IPA remains to be explained. In our animal model, results were disappointing. As with GM detection, results for PCR in diagnosing IPA in patients differ considerably between centres. This may partly be due to different PCR methods that are used. An additional problem with PCR is that it often lacks specificity. However, PCR techniques for diagnosing IPA are improving. Especially when a commercially available, sensitive technique would be on the market, PCR may be quite useful in diagnosing IPA, perhaps in combination with GM detection.

Monitoring

In animal models of IPA, progression of disease and effect of antifungal treatment is usually measured by animal survival rate. However, other parameters of fungal infection may also be useful to monitor the disease, and may increase the sensitivity in the detection of differences in efficacy between various treatment regimes.

In **Chapter 3**, we investigated a number of parameters of fungal disease for their usefulness in monitoring the disease, and measuring effect of treatment. Treatment consisted of a standard dose of amphotericin B 1 mg/kg/day for 10 days. In untreated rats, dark-red haemorhagic pulmonary lesions were seen that increased in size with progression of disease. In the left lung, cfu did not increase over time, and even showed a decrease, despite progression of disease. Left lung GM concentrations and chitin amount increased over time, as did serum GM levels. In **Chapter 4**, these serum GM levels were shown to be strongly inversely correlated with rat survival time. In treated rats, the aspect of pulmonary macroscopic lesions changed from dark-red to light-red. Histopathology of these "responsive lesions" revealed decreased broncho- and angio-invasion and decreased tissue haemorrhagia. Left lung cfu was not changed. Left lung chitin amounts, left lung GM concentrations and serum GM levels al significantly decreased under treatment.

In conclusion, cfu was not an appropriate parameter for monitoring progression of disease or measuring treatment effect. Size and aspect of pulmonary lesions, left lung GM and chitin amounts were indicators for fungal load. The most useful parameter to monitor disease progression and treatment effect was serum GM, since this parameter can be sampled serially, dissection of the animal is not necessary, concentrations are strongly associated with animal survival and levels are significantly suppressed by antifungal treatment. Most of the parameters were used in the treatment study described in **Chapter 8**.

Scintigraphic imaging

Scintigraphic imaging may have value in the early diagnosis of IPA. In contrast to X-ray and CT, it can detect physio-chemical changes, and detection of IPA is not dependent on structural changes in the lung caused by the presence of the fungus. The "gold standard" for scintigraphy of infections is the leucocyte scan, but for obvious reasons this is not feasible in neutropenic patients. In these patients, the technique that is most commonly used is de ^{67}Ga-citrate scan. In **Chapter 6**, we compared ^{67}Ga-citrate with

two new scintigraphic agents: ^{99M}Tc-PEG-liposomes and ^{99M}Tc-HYNIC-IgG in the rat model of IPA. ^{99M}Tc was used, because this substance has better imaging properties and lower radiation exposure than ^{67}Ga. PEG-liposomes and IgG were used because these substances have the property to localise at the site of infection. Both ^{99M}Tc-PEG-liposomes and ^{99M}Tc-HYNIC-IgG showed significantly higher accumulation in the infected lung than ^{67}Ga-citrate. These data suggest improved infection targeting in patients with IPA when the two newer agents are employed. ^{99M}Tc-PEG-liposomes demonstrated slightly better uptake than ^{99M}Tc-HYNIC-IgG, and therefore this agent was further evaluated in the rat model in **Chapter 7** and compared with GM detection in serum. It was demonstrated that liposomal uptake in the infected left lung increased during the course of the disease. In a very early phase of the disease, liposomal accumulation was significantly elevated and the majority of scintigrams were assessed positive, even before GM detection became positive. This suggests that scintigraphy may be positive very early in the course of the disease in patients with IPA, when structural changes caused by the fungus in the lung are still small, and X-ray and CT may therefore still be negative. A disadvantage of scintigraphy is that it is not specific for aspergillosis. Therefore, the technique should be combined with specific methods such as GM detection in BAL-fluid or transthorical biopsies. An advantage of scintigraphy is the possibility of rapid whole-body evaluation. In theory, this would allow detection of extra-pulmonary fungal disseminations. In our model we could not demonstrate enhanced liposomal uptake in sites of dissemination. However, fungal dissemination occurs late in our rat model, and therefore measurements in the early phase of the infection not demonstrate a significant difference. Hence, we consider the scintigraphic approach as being worthwhile for examination in febrile neutropenic patients.

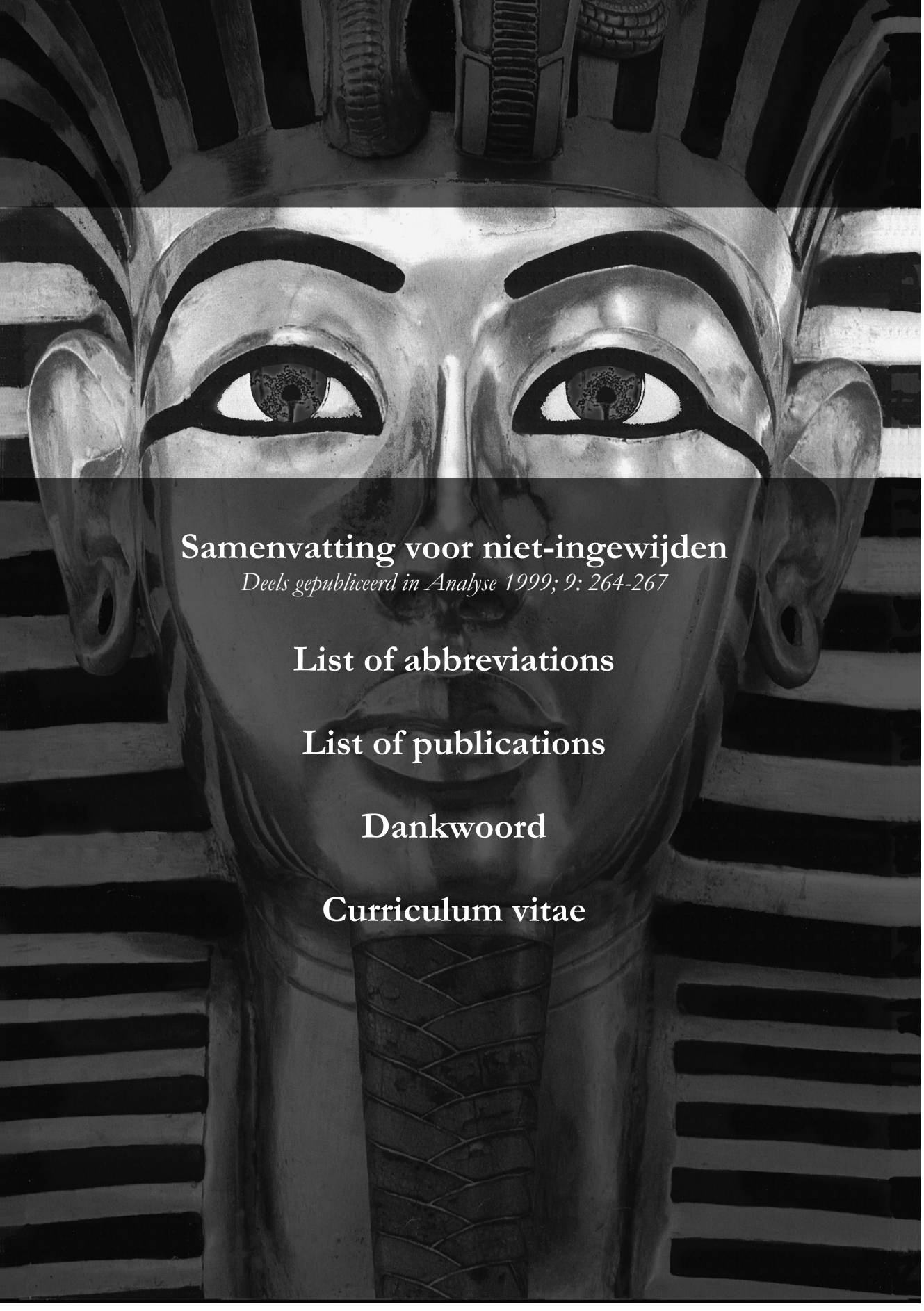
TREATMENT

Until recently, treatment of IPA with amphotericin B-deoxycholate (AMB) has been the standard treatment in many centres. As in neutropenic patients with IPA, treatment efficacy in our rat model of IPA using AMB is still suboptimal. This is partly due to the limited therapeutic range of AMB, as a result of its toxicity. Less toxic lipid-formulations of AMB have been developed, that can be administered in higher doses. Liposomal amphotericin B (L-AMB) is one of these new lipid formulations. However, L-AMB doses as high as 10 mg/kg/day did not show improved survival compared to standard 1 mg/kg AMB treatment in earlier studies in our model. Before the study described in **Chapter 8**, we tried to improve treatment efficacy by administration of even higher doses L-AMB, i.e. up to 30 mg/kg/day. However, this did not improve treatment outcome (unpublished data). It was hypothesised that the therapeutic efficacy of L-AMB could be improved by increasing the bio-availability of amphotericin B in the early phase of treatment. In **Chapter 8**, a treatment regime of L-AMB 10

mg/kg/day was compared with a regime consisting of L-AMB 10 mg/kg/day plus AMB 1 mg/kg on the first day of treatment. The addition of the single dose of AMB resulted in a significant increase in survival, probably by increasing bio-available amphotericin B.at the start of treatment. Also, left lung cfu and chitin content were significantly decreased in the combination regime.
These results may have important consequences for patients with suspected fungal infection in which treatment is started with L-AMB. It should be realised that this treatment may not be optimal when no efforts are undertaken to compensate for the delayed bio-availability of amphotericin B when administered in the liposomal form.

FINAL REMARKS

This thesis did not address one aspect of the management of IPA, which is prophylaxis. This is an important aspect of the management of IPA, since treatment regimes are still suboptimal. At the moment, we are carrying out a double-blind, placebo-controlled clinical trial, in which neutropenic patients receive prophylactic nebulisations of L-AMB, in an attempt to reduce the incidence of IPA. The advantage of using L-AMB, is that it is less toxic than AMB. The trial is performed in the haemato-oncological wards of the Erasmus MC in Rotterdam.



Samenvatting voor niet-ingewijden

Deels gepubliceerd in Analyse 1999; 9: 264-267

List of abbreviations

List of publications

Dankwoord

Curriculum vitae

SAMENVATTING VOOR NIET-INGEWIJDEN

De vloek van de mummie

Nadat in 1922 het graf van Toetanchamon ontdekt werd stierf een groot aantal mensen die de tombe hadden betreden een raadselachtige dood. De slachtoffers werden vaak binnen enkele weken na het bezoek aan het graf ziek en kregen hoge koorts. Al snel was de wereld in de ban van deze "vloek van Toetanchamon".
Vele jaren later, in 1995, maakte de Amerikaanse kunsthistorica Sheryl Munson haar droomreis: een bezoek aan Egypte en het graf van Toetanchamon. Toen ze de tombe betrad was ze volledig gefascineerd door de prachtige muurschilderingen die ze daar zag. Haar leraar had gezegd dat als ze ooit in de tombe kwam, ze dan de verf moest aanraken, want die kans zou ze geen tweede keer meer krijgen. Ze boog dan ook voorover naar de prachtige rode en blauwe schilderijen en wreef met haar hand over de verf. Drie weken na thuiskomst moest ze af en toe hoesten, hetgeen steeds erger werd. Haar man zei dat ze naar de dokter moest gaan, maar ze weigerde. Ze had een zware strijd gestreden tegen de ziekte van Hodgkin (een soort kanker van de lymfeklieren). Ze had chemotherapie gehad, ze was bestraald, alles wat je doormaakt als je kanker hebt. Ze wilde nooit meer een dokter zien, maar er was geen andere keus. Bij opname in het ziekenhuis was ze afgevallen, zwak en kortademig. Er was iets met haar longen, wat ook bleek uit een afwijkende longfoto, maar de artsen konden niet precies duiden wat het was. Het ging snel achteruit met Sheryl, en 10 dagen na opname overleed ze. Vlak voor haar overlijden werd een longbiopsie gedaan en werden de longen gespoeld. Zowel uit het longbiopt als het longspoelsel werd de schimmel *Aspergillus* gekweekt. Op basis van deze bevinding besloten onderzoekers het graf van de Farao aan een nader onderzoek te onderwerpen. Zij ontdekten dat de zwarte, afgebladderde verf op de muren van de tombe geen verf was maar een schimmel, en wel *Aspergillus*. De schimmel voelde zich prima thuis in het vochtige, warme milieu in de tombe. Het zou dus zeer goed mogelijk kunnen zijn dat Sheryl hier de besmetting met de schimmel had opgelopen. Bij nader onderzoek van de mummie bleek deze te wemelen van de bacteriën en schimmels, waaronder grote hoeveelheden *Aspergillus*. Dit zou de dood kunnen verklaren van drie mannen die in de jaren twintig de mummie in stukken hadden gehakt in een poging de kostbare amuletten die de Farao bij zich droeg van het lichaam los te wrikken (figuur 1). Mogelijk zouden hierbij grote hoeveelheden schimmel- sporen zijn vrijgekomen die vervolgens door de mannen werden ingeademd. De schimmel zou verder een verklaring kunnen zijn voor een aantal van de andere mysterieuze sterfgevallen van mensen die de tombe bezochten.

Kenmerken invasieve aspergillose

Invasieve aspergillose wordt veroorzaakt door de schimmel *Aspergillus*. Deze schimmel is algemeen aanwezig: hoge concentraties komen bijvoorbeeld voor in potgrond, vochtige kelders en stoffige ruimtes. *Aspergillus* soorten die vooral in de kliniek van belang zijn, zijn *Aspergillus fumigatus* en *Aspergillus flavus*. De schimmel is voor mensen met een normale immunologische weerstand niet pathogeen, maar in patiënten met

een verminderde weerstand kan *Aspergillus* ernstige infecties geven. Met name patiënten met een neutropenie (verlaagd aantal neutrofiele granulocyten in het bloed) lopen risico. Patiënten die behandeld worden voor een hematologische maligniteit (bloed kanker, zoals leukemie) vormen een belangrijke groep. In 80-90% van de gevallen is de long het aangedane orgaan (pulmonale aspergillose), na besmetting via het inademen van de sporen van de schimmel.

Figuur 1 De archeoloog Howard Carter onderzoekt de mummie van Toetancharnon. Later zou blijken dat de mummie hoge concentraties schimmels en bacteriën bevatte..

Ziektebeloop en behandeling

Wanneer de ziekte niet behandeld wordt is deze vrijwel altijd dodelijk wanneer de afweer gestoord is. Therapie bestaat uit het intraveneus toedienen van de antischimmel medicijn amphotericine B of voriconazol. Soms wordt ook wel itraconazol gegeven. In veel gevallen helpt de behandeling echter niet. Een belangrijke reden hiervoor is dat de diagnose vaak pas laat gesteld wordt, wat komt doordat er nog steeds geen goede test is om de ziekte in een vroeg stadium aan te tonen. Bovendien heeft m.n. amphotericine B toxische bijwerkingen en kan daardoor niet al te hoog gedoseerd worden.

DIT PROEFSCHRIFT

In het onderliggende proefschrift worden de volgende aspecten van invasieve pulmonale aspergillose (IPA) onderzocht:

- De pathofysiologie (ziekte-mechanisme) en afweerrespons van IPA werden onderzocht in een rat-model van links-zijdige invasieve pulmonale aspergillose.
- Nieuwe diagnostische technieken om de ziekte in een vroeg stadium aan te tonen werden uitgetest in het rat-model en in patiënten.
- Testen om het ziekteverloop te monitoren werden onderzocht.
- Behandeling met een nieuwe, minder toxische formulering van amphotericine B, namelijk liposomaal amphotericine B, werd geoptimaliseerd in het rat-model.

Pathologie, pathofysiologie en afweerrespons

In de **hoofdstukken 3, 6 en 7** werd de macroscopische en microscopische pathologie van de longen in het rat-model onderzocht. Macroscopisch werden donkerrode infarcten in de geïnfecteerde linker long gezien, toenemend in grootte in het verloop van de ziekte. Microscopisch werd invasieve groei van de schimmel in bloedvaten en bronchi, radiale groei van de schimmel en weefsel necrose gezien. Deze kenmerken komen overeen met die van patiënten met IPA, en suggereren daarom dat het diermodel geschikt is voor de bestudering van de pathofysiologie van IPA.

Pathofysiologie

Hoofdstuk 2 beschrijft de pathofysiologie van IPA in het rat-model, met de nadruk op de longfunctie. In het verloop van de ziekte lieten de ratten met IPA een daling in lichaamstemperatuur en gewicht zien. Tevens werd een toename gezien in "respiratoire distress" (bemoeilijkt ademhalen), wat een verminderde longfunctie suggereerde. Compliance (long-elasticiteit) en functie van surfactant (oppervlaktespanning verlagende stoffen in de longblaasjes) bleken verminderd in de geïnfecteerde linker longen van ratten met IPA. Echter, zuurstof en CO_2 spanning in het bloed bleken niet verlaagd te zijn. Kennelijk was de niet-geïnfecteerde rechter long in staat te compenseren voor de verminderde functie van de geïnfecteerde linker long. De bloeddruk daarentegen was wel significant verlaagd in ratten met IPA, en dit zou dan ook een belangrijke (mede-) oorzaak kunnen zijn bij het overlijden van de ratten aan IPA.

Afweerrespons

Bij de pathogenese van infecties spelen cytokines en chemokines (signaal-stofjes tussen cellen van het immuunsysteem) een belangrijke rol. In **hoofdstuk 3** werden verschillende cytokines waarvan gedacht wordt dat ze wellicht een rol spelen bij IPA gemeten in de longen en bloed van ratten: de pro-inflammatoire (vroeg in de infectie

uitgescheiden) cytokines TNF-α en IL-1β, de T-helper cel 1 (Th1, bepaald subtype witte bloed cellen) geassocieerde cytokine IFN-γ, de Th2 geassocieerde cytokines IL-4, IL-6 en IL-10 en de chemokines (stoffen die bepaalde witte bloed cellen kunnen aantrekken) MIP-2 en MCP-1. De pro-inflammatoire cytokines waren niet verhoogd in ratten met IPA. Dit is in tegenstelling tot de situatie bij veel bacteriële infecties. Kennelijk was de sterke bloeddrukdaling in ons rat-model **(hoofdstuk 2)** onafhankelijk van deze cytokines en spelen wellicht andere stoffen (bv toxische stoffen, uitgescheiden door de schimmel) een rol. Van de andere cytokines was alleen IL-6 in de geïnfecteerde long verhoogd, duidend op een Th2 respons, zoals ook in andere diermodellen van IPA eerder is beschreven. Ook MCP-1 en MIP-2 waren verhoogd, wat mogelijk duidt op een poging van het afweersysteem om meer witte bloedcellen naar de plaats van de infectie aan te trekken.

Diagnostiek, monitoring en scintigrafische beeldvorming

Diagnostiek

In **hoofdstuk 4** werden twee nieuwe, veelbelovende, technieken voor de diagnostiek van IPA uitgetest in het rat-model: de PCR (polymerase-ketting reactie, waarmee DNA van de schimmel kan worden aangetoond) en galactomannan (GM) detectie (GM is een bestanddeel van de celwand van de schimmel en kan d.m.v. een antistof-test worden aangetoond). In vergelijking met de PCR, was GM detectie in bloed bij meer ratten positief, en was deze test ook vroeger in het ziekteverloop positief. Ook in BAL-vloeistof (longspoelsel) was GM detectie vaker positief en beter geassocieerd met invasieve schimmelgroei dan PCR.

Hoofdstuk 5 beschrijft de evaluatie van de GM detectie-test in leukemie patiënten die chemotherapie kregen, en daardoor een verhoogd risico hadden op IPA. Ondanks regelmatig afname (2 maal per week) van bloed bij deze patiënten vielen de resultaten tegen: bij slechts 47% van de patiënten met IPA was de test positief. Daarentegen was de gevoeligheid de test in BAL-vloeistof van de patiënten met IPA veel hoger, nl 90%. Als gevolg van deze studie wordt de GM test nu routinematig uitgevoerd in de BAL-vloeistof van patiënten die verhoogd risico lopen op IPA.

Monitoring

In dier-modellen van IPA, wordt de progressie van de ziekte en de effectiviteit van therapie over het algemeen gemeten door het meten van de overleving van de dieren. In **hoofdstuk 3** werden verschillende andere parameters om de ziekte te monitoren onderzocht in het rat-model: laesiegrootte en aspect van de geïnfecteerde long, aantal cfu (aantal schimmel-kolonies dat groeit na fijnmalen geïnfecteerde long), GM concentratie in bloed en geïnfecteerde long, en chitine hoeveelheid in de geïnfecteerde long. Behalve het aantal cfu, namen alle parameters toe met voortschrijden van de ziekte. Standaard therapie met amfotericine B deed alle parameters, behalve aantal cfu,

afnemen. Concluderend was het aantal cfu geen goede parameter om de ziekte te monitoren en de andere parameters wel. De meeste van de genoemde parameters werden gebruikt in de therapiestudie beschreven in **hoofdstuk 8.**

Scintigrafische beeldvorming
Bij scintigrafische beeldvorming worden radioactieve stoffen bij een patiënt met en infectie-verdenking ingespoten. Wanneer de radioactiviteit dan ophoopt op de plek van de infectie, kan deze met behulp van een speciale camera in beeld gebracht, en daarmee gelokaliseerd worden. In **hoofdstuk 6** hebben we 3 verschillende radioactief gelabelde stoffen met elkaar vergeleken en gekeken welke van de stoffen het beste lokaliseerde ter plaatse van de infectie in het rat-model van IPA. Onderzocht werden ^{67}Ga-citraat (radioactief Gallium), ^{99M}Tc-PEG-liposomen (radioactief gelabelde liposomen) en ^{99M}Tc-HYNIC-IgG (radioactief gelabelde antistoffen). Van de liposomen en antistoffen werd gehoopt dat deze beter ter plaatse van de infectie zouden lokaliseren dan het tot nu toe meest gebruikte Gallium. Dit bleek inderdaad zo te zijn: beide stoffen lieten een hogere opname in de geïnfecteerde long zien dan ^{67}Ga-citraat, en ^{99M}Tc-PEG-liposomen werden iets beter opgenomen dan ^{99M}Tc-HYNIC-IgG. In **hoofdstuk 7** werd scintigrafie met ^{99M}Tc-PEG-liposomen verder geëvalueerd in het rat-model en vergeleken met GM-detectie. Er werd gevonden dat opname van de liposomen in de geïnfecteerde linker long toenam met progressie van de ziekte. Verder was reeds in een vroeg stadium van de ziekte de liposomale opname significant verhoogd en werd dit ook zichtbaar op scintigrammen (radioactiviteit-foto's) van de longen, zelfs voordat GM in het bloed positief werd. Dit geeft aan dat scintigrafie vroeg in het verloop van de ziekte positief wordt, en dat zou wellicht ook het geval kunnen zijn bij patiënten met IPA.

Therapie

Tot voor kort was amfotericine B (AMB) vaak de standaard therapie bij IPA. Echter, AMB kan niet te hoog worden gedoseerd vanwege toxiciteit, en daarom zijn andere, minder toxische formuleringen van AMB ontwikkeld die hoger gedoseerd kunnen worden, zoals liposomaal AMB (L-AMB). Echter, ook wanneer L-AMB in hogere doseringen gebruikt wordt zijn er nog steeds patiënten met IPA die niet genezen. Ook in het rat-model van IPA leidde het toedienen van hoge doses L-AMB slechts tot overleven van een deel van de ratten. In **hoofdstuk 8** werd in het rat-model onderzocht of de effectiviteit van L-AMB verbeterd kon worden door dit middel te combineren met AMB. De hypothese hierachter was dat de biologische beschikbaarheid van amfotericine B, wanneer toegediend via de liposomale vorm, in het begin van therapie laag zou zijn door het langzaam vrijkomen van amfotericine B uit de liposomen. In **hoofdstuk 8** werd een behandelingsschema van L-AMB 10 mg/kg/dag gedurende 10 dagen vergeleken met een schema van L-AMB 10 mg/kg/dag gedurende 10 dagen plus AMB 1 mg/kg op dag 1 van therapie. Het resultaat van de toevoeging

van AMB op dag 1 was een significante toename in overleving en een significante daling in het aantal cfu en chitine hoeveelheid in de geïnfecteerde linker long. De meest waarschijnlijke verklaring voor deze verbetering in therapie resultaat was dat het toevoegen van AMB aan L-AMB aan het begin van therapie de biologische beschikbaarheid in de long bij start van therapie verhoogt.

LIST OF ABBREVIATIONS

AMB	amphotericin B
BAL	broncho-alveolar lavage
BMT	bone marrow transplant
cfu	colony forming unit
CT	computed tomography
DOC	desoxycholate
^{67}Ga	^{67}Gallium
GM	galactomannan
IA	invasive aspergillosis
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
IPA	invasive pulmonary aspergillosis
i.v.	intravenous
L-AMB	liposomal amphotericin B
MCP	monocyte chemoattractant protein
MIC	minimal inhibitory concentration
MIP	macrophage inflammatory protein
NPV	negative predictive value
PCR	polymerase chain reaction
PEG	polyethylene glycol
PPV	positive predictive value
^{99M}Tc	^{99M}Technetium
Th	T-helper cell
TNF	tumor necrosis factor
spp.	species

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CURRICULUM VITAE

Martin Becker werd geboren te Baarn op 5 mei 1968. Het VWO diploma werd behaald aan het Eemland College in Amersfoort in 1986. Hetzelfde jaar startte hij met de studie Medische Biologie aan de Rijks Universiteit Utrecht, welke in 1992 werd afgerond. Aan dezelfde universiteit werd in 1990 aangevangen met de studie Geneeskunde. Deze studie werd onderbroken door de militaire dienstplicht (1993-1994) waarbij de functie van wachtmeester-verkenner bij de veld-artillerie werd vervuld. Het arts examen werd behaald in 1997. Eind 1996 werd aan de afdeling Medische Microbiologie en Infectieziekten van het Erasmus MC als AIO gestart met het onderzoek dat tot het huidige proefschrift leidde, onder directe begeleiding van Dr. I.A.J.M. Bakker-Woudenberg en Dr. S. de Marie. In 1998 werd begonnen met de opleiding tot arts-microbioloog, aan dezelfde afdeling (opleider: Prof. dr. H.A. Verbrugh).
Sinds 1999 is hij getrouwd met Nurma Nur Rochmah. In 2003 werd hun zoontje Thomas geboren.