Pathophysiology of unilateral pulmonary aspergillosis in an experimental rat model


*Department of Medical Microbiology and Infectious Diseases, and †Department of Anesthesiology, Erasmus MC – University Medical Center Rotterdam, The Netherlands

Because little is known about the pathophysiology of invasive pulmonary aspergillosis (IPA), we examined changes in pulmonary and general physiology during this disease in an animal model. 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 nine rats with IPA was used for measurements of arterial blood pressure, arterial O₂ and CO₂ pressure, lung compliance and surfactant function. 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 O₂ and CO₂ pressures were not different between rats with IPA and uninfected controls. Infection with Aspergillus 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.

Keywords Pathophysiology, pulmonary, aspergillosis, rats

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-characterized 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 necrotizing pneumonitis characterized by hyphal proliferation in the pulmonary parenchyma and invasion of pulmonary bronchi and blood vessels, resulting in thrombosis and hemorrhagic infarction. Hematogenous 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 comorbidity 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 characterized 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 [4,5]. 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.

Strain of Aspergillus fumigatus and preparation of inoculum
The strain of Aspergillus fumigatus that was used in all experiments was obtained from a hemato-oncological patient with invasive pulmonary aspergillosis. The patient developed the disease during a neutropenic episode after chemotherapy. The strain was isolated from a lung-biopsy of the patient and identified by culture and microscopy. During experiments the strain was kept on sabouraud medium (Oxoid, Basingstoke, UK) under oil and once every month, it was passed into neutropenic rats to maintain its virulence. To obtain conidia for experiments, the strain was cultured at 37°C for 96 h on Sabouraud dextrose agar until dense mycelium with abundant conidia had formed. Conidia were removed from the thallus by washing the colony surface with phosphate-buffered saline. This resulted in a conidial suspension without hyphal elements (microscopally checked.) The concentration of the inoculum was prepared by counting the conidia in a hemocytometer.

Infection model of IPA
Our infection model of invasive pulmonary aspergillosis 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 x 10⁹/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 anesthetized with fentanyl citrate (Hynorm, 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 specifically inoculated with 6 x 10⁴ A. fumigatus conidia in 0.02 ml phosphate buffered saline (PBS, pH 7.4). This resulted in a left-sided IPA. Survival of rats was monitored twice daily.

Clinical parameters
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. Clinical parameters were measured as described before [6].

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 anesthetized 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 O2 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 [7]. Maximal compliance (Cmax) was defined as the steepest part of the pressure-volume curve [8].

Broncho-alveolar lavage (BAL) was performed by separate lavage of the infected left and uninfected right lung with 5 ml saline-CaCl2 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 [9]. 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 three 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 (Fig. 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 (Fig. 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.

The average body weight of naive rats remained about constant over time (Fig. 3). The initial body weight of neutropenic, uninfected rats was slightly lower than that of 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 (Fig. 4). Severe respira-
tory distress had a high positive predicting value (80%) for death within 24 h. However, four rats (21%) died without signs of respiratory distress at any time during the disease process.

Wheezing was observed in a minority of rats (Fig. 5). The predictive value of wheezing for death within 24 h was 100%.

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 < 0.003 for both comparisons). All neutropenic rats with IPA showed signs of moderate or severe respiratory distress at day 7 (Fig. 4). Despite this respiratory distress, the arterial O₂ pressure was not decreased in the infected rats. In addition, the arterial CO₂ 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.

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. We used a unilateral model because this model resembles more closely the clinical situation in patients in which aspergillosis often first starts in one lung, and not in two lungs at the same time (although dissemination to the contra-lateral lung of patients occurs, as can occur in our rat model). Also, our unilateral model allows comparison between an infected and uninfected lung in the same animal.

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 [5,10].

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

<table>
<thead>
<tr>
<th></th>
<th>Neutropenic rats with IPA</th>
<th>Neutropenic uninfected rats</th>
<th>Naive rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>9</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Systolic arterial blood pressure (mm Hg)</td>
<td>71.2 ± 5.3*</td>
<td>105.4 ± 3.1</td>
<td>125.8 ± 7.1</td>
</tr>
<tr>
<td>Diastolic arterial blood pressure (mm Hg)</td>
<td>57.0 ± 5.3*</td>
<td>87.0 ± 1.9</td>
<td>104.4 ± 5.9</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mm Hg)</td>
<td>62.6 ± 5.6*</td>
<td>117.0 ± 2.3</td>
<td>125.8 ± 7.1</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
<td>96.3 ± 9.6</td>
<td>87.5 ± 7.7</td>
<td>91.5 ± 6.2</td>
</tr>
<tr>
<td>PaCO2 (mm Hg)</td>
<td>49.2 ± 9.1</td>
<td>51.2 ± 5.2</td>
<td>50.7 ± 4.9</td>
</tr>
<tr>
<td>Weight infected left lung (g)</td>
<td>1.40 ± 0.14*</td>
<td>0.68 ± 0.06</td>
<td>0.70 ± 0.05</td>
</tr>
<tr>
<td>Weight uninfected right lung (g)</td>
<td>1.28 ± 0.09</td>
<td>1.26 ± 0.12</td>
<td>1.14 ± 0.08</td>
</tr>
<tr>
<td>Macroscopic pulmonary lesion size (%)</td>
<td>67.9 ± 9</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Cmax (ml/cm H2O * kg)</td>
<td>3.57 ± 0.23*</td>
<td>6.67 ± 0.2</td>
<td>6.39 ± 0.24</td>
</tr>
<tr>
<td>Minimal surface tension (mN/m) infected left lung</td>
<td>30.9 ± 2.0f</td>
<td>19.7 ± 2.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>Minimal surface tension (mN/m) uninfected right lung</td>
<td>18.1 ± 1.4</td>
<td>18.7 ± 2.9</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

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.

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 [11,12]. 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 cryesthesia may be an alternative to fever for coping with infections [13].

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 [14,15].

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 [16]. However, the accumulation of plasma proteins in the alveoli, dose dependently impairs the endogenous surfactant system [7]. 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 compartmentalization is preserved during unilateral pneumonia [17], whereas others found that unilateral pulmonary infections could cause contralateral lung damage by induction of cytokines and neutrophil influx [18].

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 [19]. 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 shunt flow through the diseased left lung and only flow hypoxic pulmonary vasoconstriction resulting in no-
in the disease. This is probably accomplished by loss of function of the left lung, at least until a late stage
of neutropenic, infected rats with IPA compensates the
unilateral lung pathology was present.

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 [21]. The cytokine TNF-α is thought to play an important role in the circulatory dysfunction, increased microvascular permeability and metabolic derangements [22,23]. 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 [24,25]. 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 [11], and earlier investigations in our rat model of IPA did not reveal increases of TNF-α in lung or serum [26]. 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 Aspergillus fumigatus in neutropenic rats resulted in hypothermia, loss of body weight and respiratory distress. Infection caused changed lung mechanics and surfac-
tant 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.

References


