Clinical signs predicting imminent death in a rat model of invasive pulmonary aspergillosis

M. J. Becker, S. de Marie & I. A. J. M. Bakker-Woudenberg
Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands [e-mail: becker@kmic.fgg.eur.nl]

Summary

To identify the terminal phase of invasive pulmonary aspergillosis in an animal model, it was investigated whether imminent death could be predicted by clinical signs. In the model, rats with a cyclophosphamide-induced neutropenia were inoculated with *Aspergillus fumigatus* conidia in the left lung, resulting in a left-sided invasive pulmonary infection with 90% mortality on day 9 after inoculation. Six clinical parameters were evaluated for their value in predicting death within 24 h: body temperature, activity, nasal discharge, body weight loss, respiratory distress and wheezing. Multivariate analysis of all the parameters revealed that the parameters that were most significant in predicting death were wheezing ($P = 0.0001$), nasal discharge ($P = 0.04$), respiratory distress ($P = 0.05$) and decrease in body temperature ($P = 0.07$).

Invasive pulmonary aspergillosis (IPA) is a major hazard for immunocompromised hosts, particularly for patients with cytotoxic agent-induced neutropenia and transplant patients receiving long-term, high-dose immunosuppression therapy. Morbidity and mortality rates in patients with IPA are still high, because of the difficult diagnosis and treatment (Andriole 1996). This is why there is an urgent need for new diagnostic techniques and new treatment regimens. Clinical reaearch is hampered by difficulties in identifying patients with proven IPA. Consequently, animal models of IPA may be helpful in the investigations to improve the diagnosis and management of this disease.

In our laboratory we have developed an animal model of IPA that closely mimics the human disease: rats receive cyclophosphamide injections resulting in persisting neutropenia and are infected with *Aspergillus fumigatus* conidia in the left lung. This infection results in 90–100% mortality if left untreated (Leenders *et al*. 1996). In this model the pathogenesis, diagnosis and therapy of IPA can be investigated. Unlike in human patients, in animal models the clinical signs are under-utilized and yet they can give additional information in monitoring the disease and evaluating the outcome of treatment (Wichterman *et al*. 1980, Bradfield *et al*. 1992). Consequently, we investigated the clinical signs in our rat model of IPA in order to identify those clinical markers predicting imminent death. With these we should be able to relate the clinical status to the diagnostic parameters in future studies.

Materials and methods

Laboratory animals

Female RP strain albino rats were bred in our own facilities and were specified pathogen free. They were maintained under standard conditions: acidified tap water (pH 3.0) and food (Hope Farms AMII, Woerden, The Netherlands) *ad libitum*; a 12:12 h light : dark cycle (lights on 07:00 h); temperature of 22°C; a relative humidity of 40–60%; a minimum of 15 air changes per hour. The animals were housed in polycarbonate cages

($40 \times 25 \times 15$ cm, l × b × h) in groups of four. Rats were used in the experiments when they were 18–25 weeks old and weighed 185–225 g. Just before infection, the animals were transferred to an animal room especially designed for infectious disease studies, in which the entrance was restricted to persons wearing gloves, masks, surgical caps and clean coats. Here the rats were housed individually in filtertop cages (polycarbonate; $30 \times 15 \times 13$ cm, l × b × h) and the cages, animal bedding, food and water were sterilized before use.

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. The animal model that we used is the one described by Leenders *et al*. (1996) with some modifications.

Induction of neutropenia

Neutropenia was induced by a single dose of cyclophosphamide (Sigma-Aldrich Chemie, Steinheim, Germany) 75 mg/kg by intraperitoneal injection 5 days before infection. This was followed by repeated doses of cyclophosphamide 60 mg/kg i.p. at 1 day before and 3, 7, and 11 days after inoculation. This protocol resulted in granulocyte counts of less than 10^8/l on the day of inoculation.

Antibacterial prophylaxis

To prevent bacterial superinfection, the 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 intramuscular injections of amoxycillin (40 mg/kg/day) were also given for the remainder of the experiment.

Fungal strain

In all experiments the same clinical isolate of *Aspergillus fumigatus* was used, isolated from an immunocompromised patient with invasive pulmonary aspergillosis. Once every month, the strain was passed into rats to maintain its virulence.

Infection of the left lung

Infection of the left lung was established according to the method described by Bakker-Woudenberg *et al*. (1982). Under general anaesthesia with Hypnorm (0.1 ml/rat, intramuscular injection, Janssen Pharmaceutical Ltd, Oxford, UK) together with Nembutal (0.3 ml/rat of a 4 × diluted solution in saline intraperitoneally, Sanofi Sante BV, Maassluis, The Netherlands), the left main bronchus was intubated, and a cannula was passed through the tube into the left lung which was then inoculated with 2×10^4 *Aspergillus fumigatus* conidia. This resulted in a one-sided invasive aspergillus pneumonia, with a mortality of about 50% on day 7, and 90–100% on day 12 after inoculation. In around 50% of the rats, fungal dissemination to other organs, especially the liver, occurred.

Clinical parameters

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). The transponders were implanted subcutaneously with the help of a specially designed 'insertor'. According to the manufacturer, the temperature could be read with a 0.5°C accuracy, and a resolution of 0.1°C in the calibrated range of 32–43°C. At the start of experiments, the *in vivo* measurements of the transponders were checked by comparing them with rectal thermometer measurements. The measurements were divided into four categories: a temperature above 36°C was designated as category 0; 35–36°C as category 1; 34–35°C as category 2; and below 34°C as category 3.

Activity was estimated by the observer on a truncated scale: 0 (normal activity), 1 (moderate activity) and 2 (inactive).

Nasal discharge was defined as the presence on the snout of the rats of red-brown

pigmented secretion, produced by the Harderian gland (Santos & Carlini 1988, Olcese & Wesche 1989). This was scored as 0 (absent), 1 (doubtful) or 2 (clearly present).

Body weight loss was defined as the loss of body weight in grammes in the preceding 24 h.

Respiratory distress was scaled into four categories. Normal breathing (frequency 85–110/min) was designated as category 0. Light respiratory distress was seen as a slightly impeded ability to expand the thorax and designated as category 1. Moderate respiratory distress was seen as a clearly impeded ability to expand the thorax and designated as category 2. Severe respiratory distress was seen as a strongly impeded ability to expand the thorax ('gasping') with reduced respiratory frequency (30–75/min) and designated as category 3.

Wheezing was defined as an audible breathing sound, mostly a squeaking sound. This parameter was scored on a scale as either present (score: 1) or absent (score: 0).

Statistical analysis: multivariate analysis was done on clinical parameters using Cox-regression (Cox 1972) to determine their significance for predicting imminent death within 24 h in the rat model.

Results

A group of 38 persistently neutropenic rats was inoculated with *Aspergillus fumigatus* into the left lung. This resulted in a lethal infection with 50% mortality on day 7 after inoculation and 90% mortality on day 9 (Fig 1). Five control rats received cyclophosphamide, but were not infected. Clinical observations were made once daily, starting on day 3 after inoculation until day 8. The choice for the six parameters was based on earlier experience with our rat model. The clinical signs were divided into two groups: general clinical signs (body temperature, nasal discharge, activity and body weight loss) and pulmonary-associated signs (respiratory distress and wheezing). For every parameter, its value for predicting death within 24 h was evaluated.

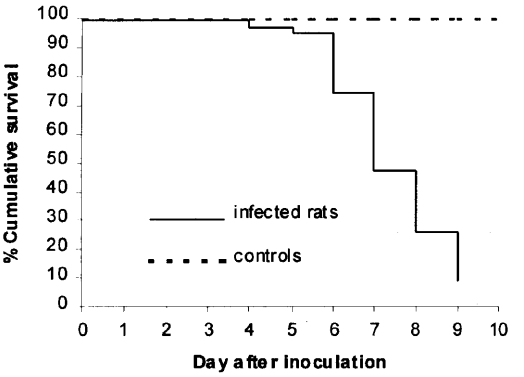

Fig 1 Kaplan–Meier plot of rats with invasive pulmonary aspergillosis ($n = 38$) and uninfected controls ($n = 5$)

General clinical signs

The average body temperature in healthy rats was 36.9°C (range 36.1–37.6°C). No apparent increase in body temperature was observed in the rats developing infection. In severely ill rats a decrease in body temperature was observed in general. When body temperature dropped beneath 34°C, nearly all the rats died within 24 h (positive predictive value (PPV) 89%, see Table 1). However, a considerable number of the rats (20%) died within 24 h with temperatures still above 36°C (Fig 2).

Inactivity was a clinical sign emerging early in the course of the disease (Fig 3). This sign had a high sensitivity (90%), but a low PPV (37%), because it occurred in many of the animals living longer than 24 h after the onset of this sign.

Nasal discharge (Fig 4) was seen as the presence on the snout of the rats of a red-brown pigmented secretion. Healthy rats would normally remove this secretion by grooming, whereas the sick rats did not, leaving the secretion visible. This clinical sign had a relatively high sensitivity (75%) and specificity (83%), but a low PPV (54%; see Table 1).

Body weight loss was a prominent feature in sick animals, but had no predictive value for death within 24 h; it was also seen in uninfected control animals. This parameter was excluded from further analysis.

Table 1 Positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity of clinical parameters for predicting death within 24 h

Clinical parameter	PPV	NPV	Sensitivity	Specificity
Temperature $< 36°C$	55%	97%	89%	80%
Temperature $< 35°C$	73%	89%	58%	94%
Temperature $< 34°C$	89%	86%	42%	99%
Inactivity	37%	96%	90%	57%
Obvious nasal discharge	54%	93%	75%	83%
Light respiratory distress	80%	90%	60%	96%
Moderate respiratory distress	100%	90%	60%	100%
Severe respiratory distress	100%	82%	15%	100%
Wheezing	73%	89%	55%	95%

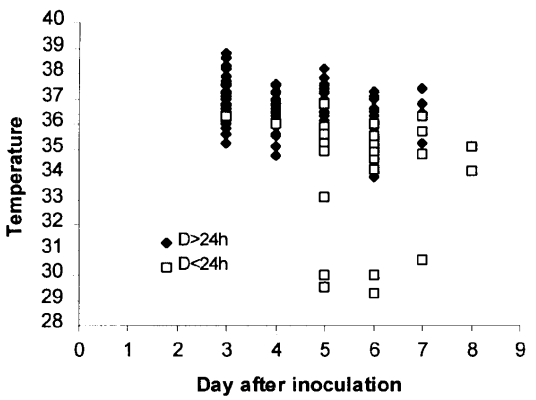

Fig 2 Temperature in rats with invasive pulmonary aspergillosis. D > 24 h = measurements in rats living longer than 24 h after measurement;
D < 24 h = measurements in rats dying within 24 h

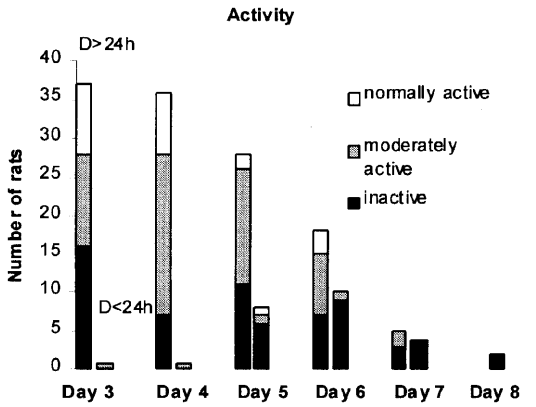

Fig 3 Activity in rats with invasive pulmonary aspergillosis. Proportions of rats in different clinical categories on day 3 to 8 after inoculation. For every day two bars are shown, the left bar indicating rats living longer than 24 h (D > 24 h), the right bar indicating rats dying within 24 h (D < 24 h)

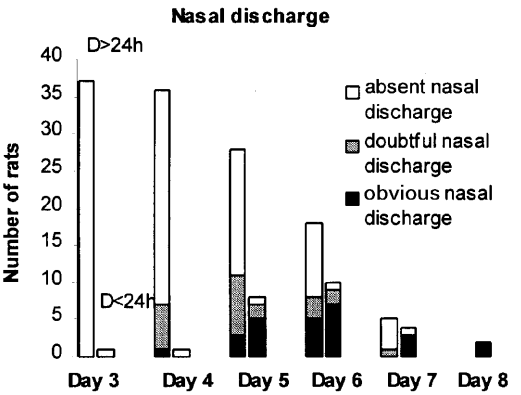

Fig 4 Nasal discharge in rats with invasive pulmonary aspergillosis. See legend for Fig 3

Pulmonary-associated signs

The respiratory rate was around 100/min in uninfected control animals. In infected animals, respiratory distress was seen as impeded breathing, and in some animals was concomitant with low breathing frequencies (30–75/min) (Fig 5). Severe respiratory distress was seen late in the course of the disease and had a very high PPV (100%, Table 1) for death within 24 h, but was only observed in a minority of the animals (sensitivity 15%).

Wheezing was defined as a clearly audible breathing sound, a typical characteristic of sick animals resulting from lung dysfunction caused by pulmonary aspergillosis. This parameter was seen in half of the rats 24 h before death (Fig 6 and Table 1).

Statistical analysis

The parameters of body temperature, activity, nasal discharge, respiratory distress and

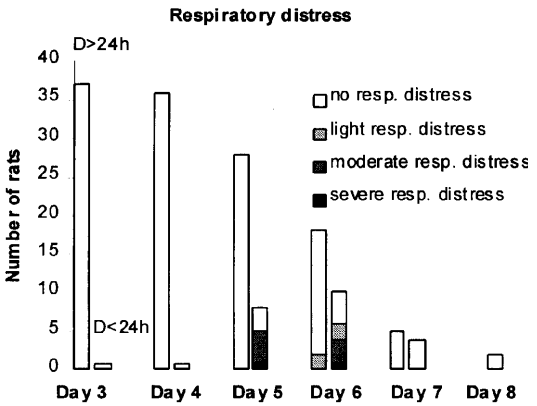

Fig 5 Respiratory distress in rats with invasive pulmonary aspergillosis. See legend for Fig 3

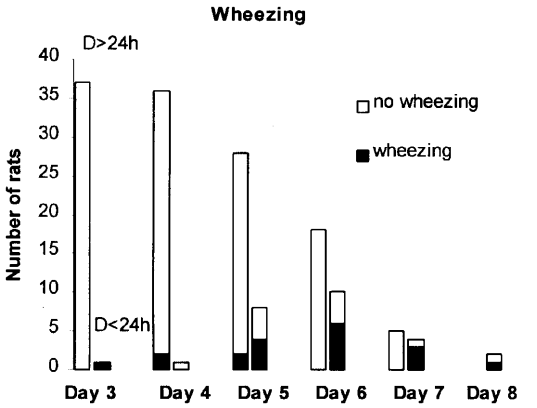

Fig 6 Wheezing in rats with invasive pulmonary aspergillosis. See legend for Fig 3

wheezing were statistically analysed using Cox regression analysis to determine their significance for predicting death within 24 h in the rat model. Table 2 shows the results of this multivariate analysis. These include the beta estimates for each variable and the degree of significance. Wheezing was the most significant parameter ($P = 0.0001$) whereas body temperature and respiratory distress gave lower P values than expected on the basis of their individual high PPV (Table 1). This was because these values were associated: animals with low body temperature in general also had respiratory distress. The parameter 'activity' was excluded from statistical analysis because of its low significance ($P = 0.37$).

Table 2 Beta estimates and *P* values of clinical parameters for predicting death within 24 h

Clinical parameter	Beta	SE (beta)	*P* value
Decrease in body temperature	0.9258	0.5168	0.07
Obvious nasal discharge	1.7294	0.8521	0.04
Respiratory distress	1.4016	0.7288	0.05
Wheezing	3.4995	0.9191	0.0001

SE = standard error

Discussion

In animal models, clinical signs are underutilized and yet can give important additional information in such experiments. For example, diagnostic parameters measured in infected animals can be related not only to the presence but also to the severity of the disease. Additionally, in some experiments it is relevant to do measurements in animals in the terminal phase of the disease, as clinical signs predicting death can then be used to determine if an animal is in the terminal phase of the disease or not, and to implement humane endpoints.

We evaluated several clinical signs for predicting imminent death in a rat model of invasive pulmonary aspergillosis: body temperature, activity, nasal discharge, body weight loss, respiratory distress, and wheezing. In our analysis we did not find any single parameter with a high fidelity for sensitivity, specificity, PPV or negative predictive value (NPV) for predicting imminent death within 24 h. Therefore, death could not be predicted in a satisfactory way using only one parameter. More than one parameter will have to be used, each with a high, independent value for predicting imminent death. Multivariate analysis revealed that the most significant parameters were: a decrease in body temperature, respiratory distress, wheezing and nasal discharge. All these parameters had a P value of less than 0.10, indicating that these parameters had considerable independent predictive value.

Other authors have also found that a decrease in body temperature predicted death in a rat model of pulmonary infection caused by *Klebsiella pneumoniae* (Kort *et al.* 1998, 1999). In this study the median survival time

of the rats, as measured with subcutaneous transponders, was 24 h when the body temperature was below 36°C. In a study of mice infected with the Venezuelan encephalomyelitis virus (Wright & Phillpotts 1998) the clinical signs of piloerection (ruffled fur), immobility and hunched posture were used as humane endpoints. However, in this study no statistical analysis was performed to correct for association between these signs. In the present study, for example, ruffled fur (data not shown) and inactivity were strongly associated with other parameters and therefore did not have a substantial independent value for predicting death.

To predict imminent death in our model, a number of parameters probably have to be combined into a clinical score. In addition, clinical parameters and scores must not only be evaluated retrospectively, but also have to be validated prospectively.

References

Andriole VT (1996) Aspergillus infections: problems in diagnosis and treatment [see comments]. *Infectious Agents and Disease* **5**, 47–54

Bakker-Woudenberg IA, van den Berg JC, Michel MF (1982) Therapeutic activities of cefazolin, cefotaxime, and ceftazidime against experimentally induced *Klebsiella pneumoniae* pneumonia in rats. *Antimicrobial Agents and Chemotherapy* **22**, 1042–50

Bradfield JF, SchachtmanTR, McLaughlin RM, *et al.* (1992) Behavioural and physiologic effects of inapparent wound infection in rats. *Laboratory Animal Science* **42**, 572–8

Cox DR (1972) Regression models and life tables. *Journal of the Royal Statistical Society* **B34**, 187–220

Kort WJ, Hekking-Weijma IM, Ten Kate MT, *et al.* (1998) A microchip implant system as a method to determine body temperature of terminally ill rats and mice. *Laboratory Animals* **32**, 260–9

Kort WJ, Hekking-Weijma IM, Ten Kate MT, Sorm V, van Strik R (1999) Determining body temperature using microchip implant system. In: *Humane Endpoints in Animal Experiments for Biomedical Research* (Hendriksen CFM, Morton DB, eds). London: Royal Society of Medicine Press, pp 122–6

Leenders AC, de Marie S, Ten Kate MT, *et al.* (1996) Liposomal amphotericin B (AmBisome) reduces dissemination of infection as compared with amphotericin B deoxycholate (Fungizone) in a rate model of pulmonary aspergillosis. *Journal of Antimicrobial Chemotherapy* **38**, 215–25

Olcese J, Wesche A (1989) The Harderian gland. *Comparative Biochemistry and Physiology* **93**, 655–65

Santos R, Carlini EA (1988) Red lacrimal secretion (chromodacryorrhea) induced by cholinergic drugs in rats subjected to the watertank technique. *Pharmacology* **36**, 61–8

Wichterman KA, Baue AE, Chaudry IH (1980) Sepsis and septic shock—a review of laboratory models and a proposal. *Journal of Surgical Research* **29**, 189–201

Wright AJ, Phillpotts RJ (1998) Humane endpoints are an objective measure of morbidity in Venezuelan encephalomyelitis virus infection of mice. *Archives of Virology* **143**, 1155–62.