Detection of Aspergillus DNA by a nested PCR assay is superior to blood culture in an experimental murine model of invasive aspergillosis

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For diagnosing invasive aspergillosis (IA), an increasing clinical problem in immunocompromised patients, molecular tools are gaining in importance. Detection of Aspergillus DNA in blood samples was investigated by a nested PCR assay in a murine model of experimentally induced IA. Ex vivo, the detection threshold of the PCR assay was determined in blood and organ homogenates of mice. After intravenous injection of Aspergillus fumigatus conidia on different days, growth of colonies was determined in cultures of blood and organs from immunocompetent and immunosuppressed mice and Aspergillus DNA was detected from blood samples by a nested PCR assay. The detection threshold of the PCR assay was as low as 1 c.f.u. ml⁻¹. The assay proved to be more sensitive than cultures of blood, with sensitivity rates between 17.6 and 87.5 % depending on the fungal burden. In conclusion, the nested PCR assay is superior to cultural methods in detecting Aspergillus spp. in murine blood samples.

INTRODUCTION

Systemic fungal infections are a frequent cause of death in immunocompromised patients, with increasing incidence and high mortality rates (Groll et al., 1996; Lin et al., 2001; Denning et al., 2003). The most frequent causative organisms, which account for more than 90 % of fungal infections in these patients, are Candida spp. and Aspergillus spp. (Bodey et al., 1992; Ruhnke & Maschmeyer, 2002). Infections with Aspergillus spp. are especially difficult to diagnose; proven diagnosis is based on histological or cytopathological detection of hyphae or positive culture results from normally sterile sites (Ascioglu et al., 2002). However, biopsy from the infected site as an invasive procedure is often not possible because patients are critically ill and have an increased risk of bleeding complications due to thrombocytopenia. Aspergillus spp. are rarely detected in blood cultures (Girmenia et al., 2001; Denning, 1998) and there are no pathognomonic signs for invasive aspergillosis (IA) on radiographic imaging. For these reasons, proof of IA is difficult to achieve.

In recent years, efforts have been made to develop and evaluate methods for non-invasive diagnostic tools for IA, such as molecular and serological techniques (Buchheidt et al., 2001; Einsele et al., 1997; Verweij et al., 1995; Maertens et al., 2001; Hanazawa et al., 2000; Makimura et al., 1994). Several trials concerning detection of Aspergillus DNA by PCR assays have been published. The clinical significance of Aspergillus PCR in diagnosing IA is unclear.

From experimental as well as clinical data, there is limited knowledge about the occurrence and duration of fungaemia and the fate of Aspergillus conidia in the organism during the course of infection.

The aim of this investigation was to compare the findings of a nested Aspergillus PCR assay with cultural results for the detection of invasive Aspergillus infection in a previously established murine model.

METHODS

Ex vivo determination of the detection threshold of the PCR assay. In an ex vivo experiment, the detection of Aspergillus DNA in blood and murine organ homogenates by a nested PCR assay was validated and the detection threshold of the PCR assay was determined.
Aspergillus fumigatus strain ATCC 9197 was cultivated on Sabouraud agar at 37 °C for 7 days. Aspergillus cultures were rinsed with isotonic saline solution and the suspension was filtered through a cell filter (Becton Dickinson) to obtain single conidia suspensions.

BALB/c mice were obtained from Harlan and used at an age of 8–12 weeks. Blood samples were gained via cardiac puncture, and organs (lungs, brain, liver, spleen) were removed under sterile conditions from non-infected, immunocompetent animals after general anaesthesia (Metofane; Jannsen-Cilag). Organs were homogenized with a Tenbroeck tissue grinder in 5 ml isotonic saline solution. One millilitre with Metofane (Jannsen-Cilag). Organs were homogenized with a Tenbroeck tissue grinder in 5 ml isotonic saline solution. One millilitre each of blood and tissue homogenates was inoculated with a defined, serially diluted number of A. fumigatus conidia (2 × 10³, 10⁴, 10⁵, 10⁶, 20, 10 ml⁻¹ and 0).

DNA was extracted by a modified phenol/chloroform extraction method from blood samples and tissue homogenates. Different amounts of the extracted DNA (0.1–500 ng) were analysed in a nested PCR assay as described previously (Składny et al., 1999). A. fumigatus DNA (0.01 ng) and human DNA (100 ng) were used as controls. To rule out contamination of blood and tissue samples during DNA preparation, DNA from a non-infected mouse was extracted and tested in the same assay. Two replicate samples at each dilution were tested.

From the number of conidia the samples were inoculated with, the amount of Aspergillus DNA was calculated in dilution series. The detection threshold of the two-step PCR assay in blood and organ homogenates was determined.

In vivo experiments. In an in vivo experiment, the detection of Aspergillus DNA by PCR assay in the blood of intravenously infected mice was compared with results from cultures of blood and organs. Both immunocompromised and immunocompetent mice were investigated.

BALB/c mice were immunosuppressed by intraperitoneal treatment with cyclophosphamide 4 days and 24 h before infection with dosages of 150 and 100 mg kg⁻¹, respectively. At the age of 8–12 weeks, immunocompetent and immunosuppressed mice were infected with 8 × 10³ Aspergillus conidia in isotonic saline by intravenous injection.

Six immunocompromised animals were sacrificed after anaesthesia with Metofane on each of days 1 and 2. Only three mice survived up to day 3. Five immunocompromised, non-infected mice served as controls. Blood samples were taken via cardiac puncture and organs were removed under sterile conditions and homogenized as described above. For cultivation, blood (200 µl) and organ homogenates (500 µl) were plated on tryptose agar and incubated at 30 °C. After 48 h, c.f.u. were counted.

Altogether 50 immunocompromised mice were sacrificed after anaesthesia with Metofane; five animals on each of days 1, 4 and 5, and seven, four and 24 mice on days 9, 25 and 30, respectively. Blood and organs were taken and further processed as described above. Seventeen non-infected mice, which were kept in the same cage as the infected mice, served as a control group.

The nested PCR assay was performed as described before by Składny et al. (1999).

Blood (200 µl) was mixed with erythrocyte lysis buffer (0.155 M NH₄Cl, 0.01 M NH₄HCO₃, 0.1 M EDTA, pH 7.4) and the mixture was incubated for 10 min at 4 °C. After lysis of erythrocytes, the sample was centrifuged at 300 g for 10 min. The supernatant was discarded, and the leukocytes were washed once with 1 × PBS (0.14 M NaCl, 5 mM KCl, 9 mM Na₂HPO₄, 2H₂O and 2 mM KH₂PO₄, pH 7.4) and re-centrifuged.

The leukocyte pellet was resuspended in 300 µl 1× PBS and the mixture was incubated with 100–125 U lyticase (Sigma) for 30 min at 37 °C to achieve degradation of fungal cells. Residual murine and fungal cell material was treated with 300–1000 µg proteinase K (Boehringer Mannheim) and 0.5 % SDS (Sigma) at 55 °C for 1 h. Residual cell material was then lysed by incubation with an additional 100 µl 2× Aspergillus extraction buffer (400 mM Tris/HCl pH 8, 1 M NaCl, 20 mM EDTA, 2 % SDS) for 30 min at 65 °C. Fungal DNA was purified by conventional phenol/chloroform extraction. The DNA was precipitated by the addition of 0.7 vols 2-propanol, pelleted, washed once with 70 % ethanol and air-dried. The DNA concentration was assessed by spectrophotometry at 260 and 280 nm.

In a two-step PCR assay, A. fumigatus DNA was amplified. The 235 bp PCR product was detected on a 2.5 % agarose gel (Składny et al., 1999).

RESULTS AND DISCUSSION

Ex vivo determination of the detection threshold of the PCR assay

In a dilution series ex vivo with different amounts of DNA used for the PCR assay, we determined the detection threshold, which was 10 c.f.u. ml⁻¹ for blood, 1 c.f.u. ml⁻¹ for brain tissue, 5 c.f.u. ml⁻¹ for spleen and 10 c.f.u. ml⁻¹ for lung and liver. This shows that our nested PCR assay described previously (Składny et al., 1999) is sensitive and is able to detect Aspergillus DNA even if the fungal load is low.

Becker et al. (2000) reported a detection limit of 10 c.f.u. ml⁻¹ in blood in a comparable experiment with rats, and Loeffler et al. (2002) achieved a detection limit of 5–10 c.f.u. ml⁻¹ in blood in their experimental model of IA using a PCR-ELISA and a LightCycler PCR assay.

In vivo experiments

In contrast to the animal models of aerogenously induced IA (Becker et al., 2000; Loeffler et al., 2002), we infected mice in our in vivo experiment intravenously to make sure that the organs of the animals were reliably infected. This is not the usual route of infection in IA, which is an airborne infection with its first manifestation in the respiratory tract (sinonasal or pulmonary IA) and secondary haematogenous dissemination. Also, the inoculum given by the intravenous route is large. Therefore, it is difficult to extrapolate from this experiment to the clinical situation. However, intravenous injection is a reliable method of reproducible induction of fungaemia and growth of fungi in parenchymal organs other than the lung in 100 % of animals, whereas, in aspergillosis induced via the respiratory tract, the occurrence of fungaemia is uncertain. Histological studies of intravenously infected mice show hyphal growth in brain and kidneys on day 2, formation of granulomas in brain, liver and kidneys on day 5 and large abscesses in the renal medulla on day 9 (Kretschmar et al., 2001).

In immunosuppressed animals, on day 1 after infection, there was Aspergillus colony growth in the blood from five of six mice (83 %), on day 2 there was colony formation in one of six mice (16.6 %) and on day 3 no colonies grew in cultures of blood. In contrast, Aspergillus DNA could be detected by PCR in the blood of all mice on days 1, 2 and 3. One reason for this could be that there was a small number of fungal elements in blood and the PCR has a lower detection...
Organ infection occurred promptly after intravenous injection of Aspergillus conidia. On day 1 after injection, growth of Aspergillus colonies could be detected in liver, brain and renal tissue cultures (Fig. 1).

Fungal elements were cleared from blood following invasion of parenchymal organs. Comparing the numbers of colonies grown from blood with colonies from tissue samples, we found a much higher fungal load in parenchymal organs than in blood only 24 h after injection of conidia, with the highest fungal loads in liver tissue. Liver tissue contains cells with phagocytosis capacity and sinusoidal cells with mannan receptors on their surface (Mori et al., 1983); this might explain the high fungal load of liver tissue. In five non-infected, immunosuppressed mice, no colony growth was observed in cultures of blood or tissue samples and PCR results from blood samples were negative.

In immunocompetent BALB/c mice, survival and follow-up were longer than in immunosuppressed animals. Cultures of blood and tissue samples from these animals were positive on day 1 after injection. Subsequently, from day 2 onwards, cultures of blood became negative in all mice. Cultures from brain tissue were positive in all mice on days 1 and 4 after infection, but only in 20% (1/5) and 14% (1/7) on days 5 and 9, respectively. Thereafter, brain cultures were negative. Liver and renal tissue showed Aspergillus colony formation in all animals up to day 25 after infection. On day 30 after infection, only 12.5% (3/24) of animals had positive culture results in liver and renal tissue. This shows that immunocompetent mice were able to clear fungi from blood and also from organs.

In the control group with 17 non-infected animals, there was no growth in cultures of blood or organs. Aspergillus PCR was negative in 15 of 17 blood samples.

We grouped mice according to results from cultures of blood and tissue samples. Blood PCR results in these different groups are shown in Fig. 2.

Of the 16 animals with positive cultures of blood and tissue samples (group a), 14 had a positive Aspergillus PCR result (sensitivity rate 87.5%). The non-infected control group (group e) consisted of 21 animals; two of these had a positive blood PCR result (specificity rate 90%). In group b, which consisted of nine mice with negative cultures of blood but positive organ cultures for all organs, the sensitivity rate of the PCR assay was 44.4%. In group d, the group with positive cultures for liver and renal tissue but negative cultures for blood and brain tissue, sensitivity was 17.6%.

It is obvious from these data that the Aspergillus PCR assay is able to detect Aspergillus DNA in blood when fungaemia is present and also in some blood-culture-negative mice, but becomes negative in a large percentage when fungal load in the organs decreases. Comparable results were found by Becker et al. (2000) and Loeffler et al. (2002), both using methodologically different PCR assays.

Loeffler et al. (2002) found positive PCR results from blood samples in only 25% of infected mice and rabbits. Becker et al. (2000) reported positive blood PCR results in 57% of rats with disseminated IA and in 27% of rats with only pulmonary IA.

From these findings, we can conclude that there is only transient fungaemia in disseminated IA with a small amount of pathogen, so that Aspergillus elements can only occasionally be found in blood samples. Still, the PCR assay was more sensitive compared to cultures of blood in detecting Aspergillus in disseminated IA.

The nested PCR assay is a feasible and specific method to

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**Fig. 1.** Mean number of c.f.u. ml⁻¹ in cultured blood or tissue on days 1 to 3 in immunocompromised BALB/c mice. Filled bars, blood; horizontally striped bars, brain; hatched bars, kidney; open bars, liver.

**Fig. 2.** Blood PCR results in different groups. a, Organ and blood cultures positive; b, organ cultures positive, blood cultures negative; c, blood and brain cultures negative, liver and kidney positive; d, organ and blood cultures negative; e, control. Open bars, PCR-negative; filled bars, PCR-positive.
detect Aspergillus DNA in blood samples of blood-culture-negative mice when fungi are present in large amounts in parenchymal organs. The sensitivity of the PCR assay varies and depends on the fungal burden and the presence of fungaemia. However, the PCR assay is superior to cultural methods because of the low detection threshold of the assay and because it detects fungal DNA independently of viability or phagocytosis.

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