Duality of liver and kidney lesions after systemic infection of immunosuppressed and immunocompetent mice with Aspergillus fumigatus

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Invasive aspergillosis is a life-threatening disease mainly caused by Aspergillus fumigatus. Patients at risk are generally immunocompromised and lungs are assumed to provide the primary site for infection and invasive disease manifestation. Contrarily, visceral organ involvement appears to result from a subsequent hematogenous spread. To compare the kinetics of dissemination within deep organs in immunosuppressed vs. immunocompetent mice, we used a bioluminescent A. fumigatus strain in an intravenous infection model. By applying an immunosuppressive regimen with corticosteroids, dissemination to the liver and kidneys was observed already 24 h after inoculation accompanied by a marked inflammatory response within the liver. In contrast, in the immunocompetent condition, fungal growth and inflammation were mainly restricted to the kidneys and only small amounts of fungal biomass and a weak inflammatory response were detected in the liver. Additionally, disease progressed much slower compared with the immunosuppressed condition. This is the first study underlying the duality between liver and renal tropism of A. fumigatus in relation to the immune status of the host.

Introduction

The rising incidence of pulmonary aspergillosis is unlikely to diminish because of the constant increase of patients with prolonged neutropenia. A. fumigatus causes a myriad of diseases ranging from non-invasive disease with superficial colonization to disseminated invasive disease with an associated high mortality rate. Immunosuppression markedly increases the risk for invasive disease characterized by tissue invasion and secondary blood-stream dissemination to multiple organs. Extra-pulmonary aspergillosis may be present in 25–60% of cases and is almost universally described in the context of disseminated diseases. Isolated extra-pulmonary aspergillosis located in the central nervous system, the skin, the liver, the urinary tract and the digestive tract has been mentioned in case reports, but potential portals of entry other than the respiratory tract are speculative.

Murine disseminated aspergillosis has rarely been documented. Indeed, mice with pulmonary infection either overcome the infection by residual immune responses in case of low dose infections or succumb from inflammatory responses or tissue infection. Thus, systemic infections using intravenous inoculation of A. fumigatus conidia have been used in immunocompetent and immunosuppressed animals to mimic the dissemination observed in humans regardless of the natural entry site of A. fumigatus. These investigations revealed that intravenous infection results in reproducible organ infection and eventual healing in immunocompetent animals.

To monitor the disease progression, two primary parameters of infection are generally followed: survival rate and fungal burden in infected tissues. In the case of invasive aspergillosis, the best suited method for determination of the infectious burden is controversial. Besides quantification of fungal genomic DNA by qPCR, determination of cell wall compounds by chitin assays, CFU determinations, or evaluation of the tissue burden from histopathologic analyses are common. All assays have in common that fungal burden can only be determined from sacrificed animals.

In this study, we addressed the following questions: (1) How does the host respond to A. fumigatus after intravenous inoculation, under immunosuppression by corticosteroids and in the immunocompetent condition, (2) what is the kinetics of disease progression, (3) does the amount of the fungal burden differ between organs and immune status and (4) what is the impact of the immune status on the inflammatory response within liver and kidneys? To answer these questions we used the bioluminescent A. fumigatus strain C3.
which constitutively expresses the firefly luciferase under the control of the gpdA promoter. In vivo bioluminescence imaging allowed studying the progression of infection in individual animals. Significant differences in the temporal and spatial manifestation of organ infections were observed in dependence of the immune status of infected animals. Results from in vivo imaging were confirmed by histopathologic analyses, showing severe inflammatory lesions only observed in the liver of immunosuppressed mice while fungal growth and infection were mainly restricted to the kidneys of immunocompetent infected mice.

Materials and Methods

Strain culturing and mouse infection. *A. fumigatus strain C3*. The bioluminescent *A. fumigatus* strain C3 was cultured on malt extract agar dexts as described earlier and bioluminescence of the strain was confirmed in vitro by determination of light emission from cultures grown for 8 h in RPMI medium by using an IVIS 100 system (Xenogen Corporation).

*In vitro bioluminescence imaging.* Male Balb/C (25 g, 6-week-old) were cared for in accordance with Institut Pasteur guidelines in compliance with the European animal welfare regulation. Immunosuppression with cortisone acetate was performed as previously described. For immunocompetent and immunosuppressed mice, two groups of five animals were infected via the lateral tail vein with 2 × 10⁶ conidia in a total volume of 100 μl.

In all experiments mice were kept for a maximum of 15 d post-inoculation. A weight curve for individual mice was recorded in 24 h intervals starting three days prior to infection.

Bioluminescence imaging and histopathology. All bioluminescence images were acquired using an IVIS 100 system as previously described. For the histopathological analyses, organs were collected between day 8 and 11 for immunocompetent mice. Organs of interest were immediately fixed in 4% neutral-buffered formalin and embedded in paraffin. Five micrometer sections were cut and stained with hematoxylin and eosin (HE) and Grocott’s methanemine silver, for detection of fungi as already described.

Statistical analysis. Comparisons between groups were performed using t tests. Significance was determined with the Wilcoxon rank test. A p value of < 0.05 was considered statistically significant. Data are reported in the figures as means ± standard deviation.

Results

Effect of the immune status on disease progression and survival. Investigating the survival of cortisone acetate treated vs. immunocompetent mice after intravenous infection with 2 × 10⁶ conidia showed significant differences in the disease patterns. All immunosuppressed animals died within three days after inoculation and lost an average of 13% of their initial body weight. In contrast, all immunocompetent animals survived the first eight days after inoculation, but started to succumb to infection the following days. Only 20% survived the observation period of 15 d accompanied by a weight loss of between 3 to 20% starting at day three after infection (Fig. 1A and B).

*In vivo bioluminescence imaging.* Bioluminescence imaging on the cortisone acetate treated animals revealed that luminescence signals were already detected from the chest and brain between 2.5 to 6 h after inoculation (Fig. 2A). After 18 h, the luminescence increased within the abdominal region suggesting that the liver became severely infected. After 28 h, the luminescence signal drastically increased with an average of total photon fluxes of 4.25 × 10⁶ from the abdominal, 1.50 × 10⁶ from the chest and 1.72 × 10⁶ from the head region (Fig. 2B).

These results clearly indicate that conidia were trapped in specific organs and possessed a rapid germination rate within these tissues, which resulted in the death of all mice within 3 d.

In contrast to the immunosuppressed animals, emergence of luminescence signals was delayed in immunocompetent animals. As seen in Figure 3A and B, unlike the low constant signal measured from head and chest areas, significant increasing signals appeared 5 d after inoculation from the abdominal region reaching peak values of 2.1 × 10⁵ and 9.5 × 10⁴ average total photon fluxes from the ventral and dorsal sides of the abdominal area at day 6 post infection (p < 0.05). In addition, as shown in Figure 1A, immunocompetent mice succumbed to infection much later than cortisone acetate treated mice (days 8–11 vs. days 2–3). Collectively, these results confirmed a higher degree of fungal growth restriction under the immunocompetent condition.

Histopathological analysis. Histopathological analyses revealed a good correlation between the luminescence signal and the presence of fungi within these tissues (Fig. 4). In the immunosuppressed condition, intravenously injected conidia massively disseminated to the liver and kidneys. In the immunocompetent condition fungal growth was mainly restricted to the kidneys and only to a very minor extent observed in the liver.

A more detailed analysis of fungal growth and histological lesions of the liver of corticosteroid treated mice revealed a marked zonal lesion centered on portal tracts and centrilobular veins, but secondly extending to sinusoids. It was characterized by large zones of hepatocyte necrosis associated with infiltration of neutrophils that were often fragmented (suppuration), lymphocytes, plasma cells, and macrophages. The cellular infiltrates often involved blood vessel walls (veins and arteries) and were associated with fibrinous thrombi (Fig. 4A). Intralesional Aspergillus hyphae were detected (Fig. 4B).

Lesions were markedly different in the immunocompetent model. In the liver of two out of five mice investigated, a minimal zonal multifocal lesion, centered on centrilobular veins, was observed (Fig. 4C). It was characterized by necrosis of hepatocytes of the centrilobular zone (Fig. 4C, inset), associated with the presence of conidia in the cytoplasm of Kupffer cells (Fig. 4D). As these conidia remained in a resting state even at late stages of infection (day 6 post-infection and beyond), it can be assumed, that Kupffer cells were able to phagocytose conidia and prevent the rapid germination within this organ.

In the kidneys of corticosteroid treated mice (Fig. 4E), a mild inflammatory lesion usually centered on cortical glomeruli was...
observed. It was characterized by necrosis of mesangial cells sometimes associated with mesangial infiltrates of lymphocytes, plasma cells and neutrophils (Fig. 4E, inset). Intralerial fungal hyphae were numerous and extended across the Bowman’s capsule (Fig. 4F).

Concerning the kidneys of immunocompetent mice, significant differences could be noted between mice that survived or not. Surviving mice indeed displayed no significant histological lesion in the kidney except one mouse with only very minimal inflammatory infiltrates in the papilla (data not shown). However,
mice that died of infection rather displayed severe lesions (Fig. 4G), centered on the pelvis and secondarily extending to renal tubes of the medulla and cortex (ascending infection: pyelonephritis), in contrast to corticosteroid treated mice, in which the cortical glomeruli were targeted (descending infection: glomerulonephritis). These lesions were characterized by a severe infiltration of neutrophils, very often fragmented (suppuration) (Fig. 4G, inset). Additionally, a high density of *A. fumigatus* hyphae was detected in the lesions of the renal pelvis (Fig. 4H).

**Discussion**

Since aspergillosis is an airborne infection, lungs are the primary organ for disease manifestation. However, other organs such as
Figure 3. Time dependent monitoring of light emission from immunocompetent mice. Luminescence was acquired from mice as indicated in Figure 2. (A) The luminescence was measured from ROIs from both ventral (V) and dorsal (D) sides. In (B) is shown the average of luminescence from head, chest and abdomen of all mice from ventral (full line) and dorsal (dashed line) sides. *p < 0.05.
the brain and the liver can become infected by dissemination through the blood stream. Although intracerebral or intravenous infections are not assumed to play an important natural route for the acquisition of invasive aspergillosis, such infections are frequently used to test the efficiency of antifungal drugs and to study the dissemination and disease progression in brain and kidneys.

Our previous studies using an intranasal infection model revealed a localization of fungi that was mainly restricted to the lung and, in some cases, the nasal sinus. Thus, to confirm that bioluminescence can be tracked from all body sites, we took advantage of intravenous conidia injections to confirm the general suitability of the system. Indeed, luminescence signals were detected from liver and kidneys and correlated with histologic analyses. Despite previous assumptions that distribution of firefly luciferin may provide a limiting factor for imaging the dissemination of infections, tracking of A. fumigatus infections from diverse body sites indicates that substrate availability is not a limiting factor of this technique.

In this study, we were additionally interested in the kinetics of fungal distribution, the velocity of the manifestation and progression of infection, the main organs targeted and the fungal load in these organs after intravenous inoculation.

An important observation from our study was the effect of corticosteroid treatment on the rapid manifestation of infection in the liver. This infection was accompanied by a moderate to marked inflammatory reaction characterized by multifocal infiltrates of neutrophils, lymphocytes and macrophages. The nature of this inflammatory infiltrate appears similar to that observed in the lungs of corticosteroid immunosuppressed mice following intranasal inoculation. In the lung of immunocompetent mice alveolar macrophages phagocytose inhaled conidia and restrict their rapid germination. This function is hampered under corticosteroid treatment resulting in a rapid escape of A. fumigatus conidia from alveolar macrophages accompanied by massive neutrophil influx with severe tissue destruction.

In the liver, nonspecific phagocytosis is mediated primarily by Kupffer cells. In agreement, in the immunocompetent condition conidia were detected in the cytoplasm of some Kupffer cells. As these conidia remained in a resting state even at late stages of infection (day 6 post-infection and beyond), it can be assumed that Kupffer cells efficiently phagocytose conidia and inhibit their germination. This is in agreement with a previous observation on A. fumigatus and Candida albicans infections in which a TLR4 independent release of proinflammatory cytokines has been observed pointing to a vital immune response of Kupffer cells toward fungal infections.

We assume that treatment with cortisone acetate might alter the efficiency of Kupffer cells to restrict fungal germination. In this respect it has been shown that high glucocorticoid concentrations reduce the production of nitric oxide, oxygen radicals and prostaglandins and impair phagocytosis. Thus, such an
impairment of function may allow the rapid germination of conidia. However, corticosteroids may also be responsible for the inflammation observed in the liver of treated animals. Systemic administration of corticosteroids increases the number of circulating neutrophils by 3- to 5-fold, leading to a significant increase in neutrophil recruitment to the site of infection. Due to the germination of conidia, the neutrophil recruitment could be further stimulated by inflammatory cytokine secretion regulated by NK and DC cells in response to fungal antigens presented on the surface of hyphae.

The second major observation concerned the kidney lesions, and more specifically the significant differences in severity and pathogenesis of these lesions between immunocompetent and immunosuppressed mice. In the immunosuppressed condition kidney lesions were characteristic for a descending infection with a glomerular capillary starting point (glomerulonephritis). These inflammatory lesions (massive neutrophil infiltration and renal parenchyma destruction) were severe enough to cause the death of infected animals. A pelvis and medulla targeting after intravenous inoculation of A. fumigatus was already described in the literature occurring 9 d after infection, but the exact mechanism of this targeting and differences concerning the immune status were not described. Our results suggest that the prolonged time course of infection in immunocompetent mice allowed the development of pelvis lesions. In contrast, the immunosuppressed mice died before any lesion could be detected in the medulla of kidneys.

Further studies are required to elucidate the exact mechanisms in kidney infections and could include other imaging techniques such as two-photon imaging. At current, such differences in kidney infections by A. fumigatus have not been described, but addressing these questions in future studies might enhance the knowledge on fungal pathogenicity mechanisms.

In conclusion, this is, to our knowledge, the first report describing the suitability of bioluminescence imaging to detect disseminated fungal infections with a qualitative assessment of the fungal burden from deep tissues such as the liver or kidneys. Although depth of infection, light absorption by hemoglobin and light scattering from bones make a transfer of light intensity signals into colony forming units difficult, this technique reflects the increase of fungal burdens in various organs from individual animals. Previous studies on disseminated candidiasis using C. albicans strains that produced either an intracellular firefly luciferase or a cell wall-bound Gaussia luciferase failed. These negative results for monitoring systemic disease were mainly attributed to limited substrate distribution of coelenterazine in case of Gaussia luciferase or a limited uptake of D-luciferin by Candida hyphae in the case of the firefly luciferase. However, our studies on A. fumigatus show that bioluminescence imaging is, in general, suitable to study disease progression of fungal pathogens and substrate availability does not appear as a major limiting factor.

Thus, this technique provides a perfect tool to identify the main target organs during an infection process and allows monitoring differences in disease progression depending on the immune status of the host. In addition, this technique provides a potential tool in prospective studies to monitor the efficacy of antifungal drugs in animal models and may be combined with additional monitoring techniques using labeled antimicrobial peptides.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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