A New Experimental Murine Aspergillosis Model to Identify Strains of *Aspergillus fumigatus* with Reduced Virulence

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Abstract

Experimental animals are an obligate screen to investigate microorganism pathogenicity. Numerous animal models have been used to analyse the virulence of the opportunistic human pathogen *Aspergillus fumigatus* but none of the experimental models used previously have been satisfactory. This report discusses these models and presents a murine model of pulmonary aspergillosis that is very easy and the most adapted to compare the pathogenicity of *A. fumigatus* strains. Strains to be tested are inoculated intranasally and synchronously to mice and strains isolated from the lung of mice killed by the infection are typed. The number of colonies recovered is directly correlated to the virulence of the strain.

Key words: *Aspergillus fumigatus*, aspergillosis, strain typing, virulence, epidemiology

Identification of putative virulence factors in *Aspergillus fumigatus* and other human fungal pathogens is based today on a two-step strategy. First, the gene encoding a putative virulence factor is disrupted and second, the infectivity of the isogenic mutant and parental strains is compared in an experimental animal model of infection.

Most of the efforts have been directed in recent years towards the construction of mutants. In contrast, less concern has been devoted to the adequacy of the experimental model used to compare mutants. Two experimental murine models of invasive aspergillosis are currently used to investigate the pathogenicity of *A. fumigatus*. The first model developed consisted in intravenous inoculations of conidial suspensions. This model differs significantly from a human invasive aspergillosis infection: (i) conidia are present in high amounts in the blood stream; (ii) infection of the mouse does not require immunosuppression; (iii) the primary target infected organ is the kidney. Its main advantage is a good reproducibility of the survival data and this model remains used to investigate the efficacy of anti-*Aspergillus* drugs. However, due to its low relevance with the pathology of human invasive aspergillosis, this iv model is often replaced by an intranasal model of infection. Under these conditions, only immunosuppressed animals can be infected and the lung is the original site of infection. The amount of conidia inhaled required to kill a mouse is negatively correlated with the severity of the immunosuppressive regimen. However, due to a variable loss of conidia in the gastrointestinal tract, accurate estimation of the concentration of spores reaching the lung is difficult to assess and the reproducibility of the infection experiments is often low. Consequently, this model has been used more to give a yes-no answer for strain pathogenicity rather than a correct quantification of the aggressiveness of various strains.

To address the problem of infection models and in addition, to mimic the hospital environment where several strains present in the same ecological niche are inhaled together by the patient, an experimental model has been developed where several strains are inoculated intranasally and simultaneously to immunocompromised mice. During this study, the infectivity of wild type strains with pigmented or white conidia was investigated. This report shows that (1) synchronous inhalation of multiple strains is a better model than single strain infection to quantify variations in strain aggressiveness; (2) wild type strains may
have a variable propensity to infect mice; (3) pigmented strains are more prone to cause infection than white strains confirming that the melanin biosynthetic pathway is associated with the virulence of *A. fumigatus*.

**Material and Methods**

**Fungal strains**

Origin of the colourless and pigmented wild-type strains of *A. fumigatus* used in this study is shown in Table 1.

**Culture conditions**

For conidial production, fungal strains were maintained on 2% malt extract agar (2%) slants for at 25°C. For germination studies, conidia recovered in PBS Tween 0.1% were deposited as a spot of 2-3 μl containing 3-4×10^3 conidia/ml on the surface of a Sabouraud (S) 2% glucose+1% mycopeptone supplemented with 2% agar and incubated at 37°C in a humid atmosphere for up to 10 hrs. For quantification of mycelial growth in liquid culture, 150 ml Erlenmeyer flasks containing 50 ml of liquid Sabouraud medium were inoculated with a suspension of 3×10^5 conidia and shaken at 200 rpm at 37°C. Two other defined liquid media were also tested: D medium containing 1% glucose, 0.5% asparagine and 0.1% KH₂PO₄ and B medium which is a Czapek medium modified by Brian. Mycelium was recovered after 24, 48 and 72 hrs by filtration, extensively washed with water and weighed after 24 hrs at 105°C.

To study the effect of cortisone, hydrocortisone (solubilised in methanol at a 12 mg/ml stock solution) or hydrocortisone 21-hemisuccinate (soluble in water and filter sterilized) was added to the D or B media at a final range of concentrations of 46, 4.6, 0.46, 0.046 μg/ml (corresponding to concentrations of 10^-4, 10^-5, 10^-6 and 10^-7 M). Germination and growth were estimated as described above.

All the germination and growth measurements were repeated at least twice.

**Scanning electron microscopy**

Fungal material was fixed with 1% OsO₄ in PBS buffer for 2-4 h at 4°C. OsO₄-treated material was post-fixed with 2.5% glutaraldehyde in PBS for 2 days at 4°C. Fixed material was washed with water, dehydrated in ethanol (20 min successive baths from 70% ethanol to 100% ethanol) and incubated 2×30 min in hexamethyldisilazane. Air-dried samples were coated with Au before observation in a Leica CamScan scanning electron microscope at 10 kV.

**DNA Fingerprinting**

DNA extraction, EcoRI digestion and southern blot hybridization with the repeated sequence λ3.9 were performed as previously described. Computerized analysis of the hybridization patterns and pairwise comparison of the densitometric profiles deduced from the southern blot hybridization patterns obtained with the 7 colourless strain and 50 randomly selected patterns of pigmented strains previously studies was done with the Gel Compar software. Southern blots were also performed using a Nat-SalI fragment of the FKS gene and a Xho-SalI fragment of the DPPV gene hybridized with total DNA digested by EcoRI, HindIII, SalI and BamHI. 32P-labelling and hybridization conditions were from standard protocols.
Infection experiments

Three colourless strains (110.46, 386.75 and 100079) and three pigmented strains (100074, 100075, 100076) were used for experimental infections. Female Swiss mice (16-18 g) were infected with either single strains or a mixture of the six strains. Two protocols of infection were used: (i) $4 \times 10^6$ conidia in saline were inoculated intravenously (i.v.) in the tail vein of non-immunosuppressed mouse recipients, (ii) $10^6$ conidia
Conidia (in 50 μl PBS Tween 0.1%) were inhaled intranasally (i.n.) to steroid-treated mice which received intraperitoneal injections of cortisone acetate (5 mg/mouse on days -3, 0, +2 and +4). With the exception of the i.v. experiment which was performed only once, all other experiments were performed at least 3 times using cohorts of 10 mice per experiment.

For mice injected with the mixture of 6 strains, 2-3 mice were euthanized 24 hours after inoculation and lungs were disrupted in a potter homogenizer in 5 ml PBS/Tween 20 0.01%; 200 μl of the lung homogenate were plated on a 2% malt +0.05% chloramphenicol medium to estimate the proportion of white and green strains present in the lung after inoculation. The remaining 7-8 mice were kept until they eventually died. On the day mice succumbed to aspergillosis, lungs were dissected and deposited on 2% agar plate containing 0.05% chloramphenicol. After 2-3 days at room temperature, the lung was covered by a white mass of mycelium that just begin to produce conidia. Two protocols were used to isolate strains from the lungs. (i) Malt slants were inoculated with mycelium obtained by a delicate contact of a loop with the sterile mycelium grown on the surface of the lung. After a week at 37°C, monospore isolates were obtained from the original slant and randomly selected isolates were fingerprinted with the λ3.9 probe as described above. (ii) Alternatively, the lung kept on the agar-chloramphenicol plates was floated on saline and

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**Fig. 2. SEM of mature conidia of white strains.**

- a: 110.46; b: 229.65; c: 100079; d: 100078; e: 386.75; f: 100077.
the conidial suspension was diluted to obtain monospore isolates directly on malt plates. Preliminary studies have shown that the 2 methods gave similar results in terms of percentage of different strains recovered. Four to 10 randomly selected strains were typed per mouse.

**Statistical analysis**

SuperANOVA software from Abacus (Abacus Concepts, Berkeley USA) was used for one and two-ways variance analysis. Statview software from Abacus was used to analyse survival data of mouse inoculated i.n., with Kaplan-Meier and Logrank (Mantel-Cox) non-parametric tests.

**Results**

**Taxonomical characterization of wild type isolates of *A. fumigatus* with colourless conidia**

Based on morphological measurements, all strains of Table 1 are typical *A. fumigatus* isolates with the exception of one criteria, the conidial pigmentation. Conidia of the *A. fumigatus* strains displayed variable surface ornamentation (Fig. 1-3). Young conidia (the youngest conidium is the
characterized by the presence of numerous spikes on their surface. When the conidia aged, the surface ornamentation changed. Conidia of the green strains showed a rougher surface than the white strains, although there was a significant variability between the different strains (Fig. 1-3). These data showed that the ornamentation of the conidial surface was depending on both the age and the presence of a pigment in the cell wall. To confirm that all pigmented and non-pigmented strains belong to the species *A. fumigatus*, the repeated DNA sequence *Afu1* which hybridizes exclusively to *A. fumigatus* DNA was used. Strong hybridization signals were obtained with EcoRI digested DNA from all colourless isolates and the λ3.9 probe (Fig. 4). Computerized analysis of southern blot patterns obtained with 7 colourless strains and with 50 randomly selected pigmented strains of *A. fumigatus* did not show any clustering of the white strains outside of the population of green strains (Fig. 5). Southern blot patterns obtained with total DNA from the 7 pigmentless and 3 green strains digested with 4 restriction enzymes and probed with fragments of *FKS* and *DPPV* genes that are present as a single copy in the *A. fumigatus* genome, were indistinguishable (see Fig. 6 as an example). Size of the hybridization bands were 4.3 kb and 0.9 kb, 8.7 kb, 10 kb and 9 kb with the *DPPV* probe and 9.4 kb, 1.0 and 1.7 kb, 1.3 and 4.3 kb and 8.0 kb for the *FKS* probe after DNA digestion with *SalI*, *HindIII*, *BamHI* and *EcoRI*, respectively. A strain of *Neosartorya fischeri*, gave different southern blot patterns with both the repeated sequence and the single copy probes (Fig. 6). All these molecular data confirmed that (i) the 7 *Aspergillus* strains with pigmentless conidia tested in this study belong to the species *fumigatus* and (ii) mutations occurring in nature in the genes of the melanin biosynthetic pathway were not associated to a specific genetic background of the mutated strains.

**Biological characteristics of the *A. fumigatus* wild type strains used in the infection assays**

Three non-pigmented (110.46, 386.75 and 100079) and 3 pigmented strains (100074, 100075 and 100076) were selected on the basis of their sporulation abilities and used for infection experiments. Comparable germination and growth kinetics were obtained with the 3 colourless and 3
pigmented strains tested (Fig. 7). In medium S, germination of strain 100074 and to a lower extent strain 100079 were slightly slower than the other strains. After 10 hours incubation, 100% of conidia from all strains germinated. The 2 white strains 110.46 and 100079 grew slightly less than the other strains. The growth differences between strains seen in medium S was also seen in defined media B and D in which strains 110.46 and 100079 grew less (data not shown).

**Virulence of* A. fumigatus* strains in mice**

Strains with hyaline and pigmented conidia were able to kill steroid-treated mice. No statistical difference in strain virulence was seen when LD 50 or survival data of mice infected by each strain inoculated separately were analysed (Fig. 8). This result suggested that either every strain tested had the same pathogenicity potential in our experimental murine model of IA or that our animal model screen was not adequate. Indeed, the extreme severity of immunosuppression regimen and the high concentration of conidia used in the in model may mask moderate differences in strain pathogenicity. To address this question, it was decided to undertake infection assays using as an inoculum a mixture of different strains.

**CFUs counts during experimental infections**

CFUs counts during experimental infections showed that alive conidia can be found in the mouse lung even a week after initial inoculation (Fig. 9). This result suggested that, eventhough a difference in the growth of the different strains present in the mixture occured in vivo, a lung collected from a mouse infected with several strains will contain alive fungal material from all inoculated strains. As a consequence, counting strains identified by PCR assays performed on total DNA lung extracts, using primers specific of every strain present in the mixture, would be inappropriate since PCR assays would be positive for all strains present in the DNA mixture, including both fast growing strains as well as “non-growing” resting or slowly growing strains.
In contrast, during a joint infection, it can be expected that the most aggressive strain that is responsible for mouse death will be growing as mycelium in the lung. This invading mycelium will emerge first through the epithelium of a lung recovered at necropsy whereas non-infective strain(s) will not be recovered (even though a non-infective strain present germinates in the lung of a dead mouse, it will come second and will not be isolated). This simple isolation procedure should allow to separate mycelium from a virulent strain actively growing in the lung tissue from non-virulent or less virulent strains that will remain as ungerminated resting conidia or mycelia with very limited extent in the lung. This working hypothesis was tested in infection assays with 3 green and 3 white strains.

When 3 pigmented and 3 colourless strains were inoculated jointly and simultaneously to mice, typing of 133 strains randomly recovered from the lung of 24 dead mice showed that the number of green strains isolated from the lung of dead mice was much higher than the number of white strains (Fig. 10). This result was even seen 24 hours after infection where 37% of white strains and 67% of green strains were isolated from lungs of mice infected for 24 hrs (data not shown). This results indicated that strains with pigmentless conidia are less virulent than strain with green conidia. Amongst the 6 strains tested in mixed infection, two strains, the white strain 110.46 and the green strain 100076 were never or very rarely recovered from the lung of dead infected mice (Fig. 11). Intravenous infection experiments gave similar results. No difference in lethality for the murine host was seen when the mice were inoculated separately intravenously with each of the 6 strains whereas strains 100076, 100.46 and 100079 strains were not recovered from infected kidneys after mixed infections (5 mice, 50 strains typed). These results suggest that strain 100076 and 110.46 are the least aggressive amongst the set of wild type strains tested. This difference of pathogenicity was not due to a differential effect of the cortisone on the growth of each strain. In contrast to previous studies it was shown that addition of cortisone at concentrations varying from $10^{-7}$ to $10^{-4}$ M did not modify the conidial germination and mycelial growth for all white or green strains tested (data not shown).
Discussion

The data reported here show that the experimental mouse model used to compare strain virulence in *A. fumigatus* is very critical. The vast majority of studies undertaken to assess *A. fumigatus* strain virulence have used single strain infection protocols. In these models, the amount of conidia required to kill the host is directly correlated to the immunosuppressive regimen. For example, 10^6 conidia per mouse are required to kill mice that have received a single cortisone acetate injection whereas only 10^4 conidia are needed to obtain the same lethality in mice successively treated with cyclophosphamide and several injections of cortisone\(^5\). In rabbit, chronic and acute infections, associated with different cellular immune responses, have been obtained through the modulation of the immunosuppressive regimen\(^10\). Such dichotomy in the patterns of infection has not been reported in mice and the development of a mouse model of chronic pulmonary infection is still expected. Indeed, the lowest inoculum dose presently used to obtain 100% mortality in immunocompromised mice i.e. 10^4 conidia remains extremely high and is equivalent to the amount of conidia breathed by a human for 1-2 month. Another model of rodent aspergillosis, recently developed, mimicks in a better way human infections\(^19\). In this model, conidia embedded in agarose are injected intratracheally into immunocompetent rats and remain dormant until mice are immunosuppressed. However, this model is not practical to test the virulence of a high number of strains. Alternatively, transgenic mice susceptible to *A. fumigatus* infections such as X-CGD mice can be infected with low amount of conidia (50 conidia per mouse)\(^21\). This inoculum seems more comparable to a more chronic clinical situation. Transgenic mice are however very difficult to obtain in high amounts to test the virulence of a lot of different strains. The model of inoculation with multiple strains used in this report seems a better screen to rank virulence levels of wild-type strains. This model can be also used to compare the virulence of mutants obtained by reverse genetics and their parental strains (author’s unpublished results). The reasons for the observed difference in pathogenicity of the different wild type strains are presently unknown.

In another experiment, one low virulent strain was inoculated 24 and 48 hours before a mixture of 5 other strains. Using the same isolation and typing protocol, it was found that earlier is the strain inoculated before the mixture of the other strains, higher is the number of isolates of this strain recovered from the lung. However, the number of isolates recovered from the most virulent strains present in the mixture of the 5 strains inoculated later was always higher than the number of isolates from the low virulent strain (data not shown). This result suggests that the differences in pathogenicity are not due to competition between strains at the time of germination but rather to a quicker growth of the virulent strains *in vivo*. Such multiple strain injections is at the basis of the assay used by Holden and co-workers to identify virulence factors in bacteria and fungi after signature tagged mutagenesis\(^27\). In their model, 96 different mutants differentially tagged are injected per mouse. However, PCR experiment performed on the lung of infected mice and the high number of mutants tested per mouse in these studies have prevented the identification of strains with lower virulence in their library of mutants. Unpublished data suggest that the number of mutants tested per mouse should be inferior to 10 to obtain reproducible infection results and best comparisons were obtained when only 2 different strains were inoculated together to the same mouse.

Melanin which results from the oxidative polymerization of phenolic precursors is present in the outer wall layer of the conidia of pathogenic species of *Aspergillus* such as *A. fumigatus*, *A. niger* or *A. nidulans*. Our study has confirmed previous studies showing that the presence of melanin in *A. fumigatus* conidia protects the fungus against external aggressions, either in natural environments or in humans when conidia are accidentally inhaled. Environmental colourless strains of *A. fumigatus* are accordingly very rarely isolated. This could be due, at least partly, to the higher sensitivity of white conidia to UV light. The 3 white strains tested (110.46, 386.75 and 100079) were killed by a 15 min exposure to UV light in our experimental conditions. Conditions of UV exposure were the following: 10^4 conidia deposited on the surface of a 2% malt agar medium were irradiated with a UV lamp (254 nm wave length, 50 Hz, 12 w VL-6MC, Bioblock, France) located 24 cm above the agar plate. Melanin has been recognized as a virulence factor amongst several human pathogenic fungi because it allows the fungus to resist the host defence mechanisms. Mortality in mice challenged with melanin deficient strains of *Cryptococcus neoformans* and *Wangiella dermatitidis* was strikingly lower than that seen in mice challenged with the parental melanin-positive strains\(^25\). The melanin present in the cell wall provides resistance against environmental stress including superoxide anion, singlet
oxygen and hypochlorite. Several authors have suggested that melanin can act as a shield against immunologically active cells and in particular protects the conidia against the reactive free radicals and lysozomal enzymes of the polymorphonuclear neutrophils and macrophages. This polycyclic aromatic pigment can also suppress lymphoproliferation and TNFα production and reduce binding to the complement C3 factor. Even though melanin has an effect on the pathogenicity of A. fumigatus, this role does not seem essential since pigmentless strains are all able to kill mice when injected intranasally and during a mixed infection a white strain like strain 386.75 may be found at a higher level than the green strain 100076. The fact that infective mycelia produced by both pigmented or non-pigmented conidia are hyaline suggest also that the lower pathogenicity of the white strains is not only due to the higher susceptibility of the pigmentless conidia to the host phagocytes but is also seen at the mycelial level. This result suggest that disruption of genes in the melanin biosynthetic pathway may be associated to the perturbation of metabolic pathways essential for mycelial growth in vivo.

References


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