MYCOLOGY

Variation in virulence of *Aspergillus fumigatus* strains in a murine model of invasive pulmonary aspergillosis

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The diversity in virulence of different *Aspergillus fumigatus* strains was studied in an experimental murine model of invasive pulmonary aspergillosis (IPA) and the results were correlated with possession of a putative molecular marker of virulence. Seven strains from different patients with non-invasive or invasive aspergillosis and four environmental strains were typed by PCR with specific primers and scored as positive or negative, according to whether or not a 0.95-kb DNA fragment was amplified. Immunosuppressed mice were inoculated intranasally with *A. fumigatus* conidia from these different strains. The mortality curves revealed differences in virulence between the strains. The environmental strains produced a weaker infection than the strains from patients and the 0.95-kb-positive patient strains caused significantly higher mortality rates in mice than the 0.95-kb-negative patient strains. These findings support the hypothesis that certain isolates of *A. fumigatus* are more virulent than others and that their virulence appears to be associated with the 0.95-kb molecular marker.

Introduction

Aspergillus fumigatus is ubiquitous in the environment and its conidia are inhaled frequently by many normal, healthy people, yet only rarely does invasive aspergillosis develop. Nevertheless, this common saprophyte is life-threatening for immunocompromised patients and the gravity of disease depends particularly on the host's phagocytic defences. However, the key events involved in pathogenesis of the infection are not yet known. It has been suggested that the production of serine and metallo-proteases plays a role in the pathogenicity of *A. fumigatus* [1–4]. However, the most recent data have suggested that these enzymes are not major virulence factors [5–8]. Recently, other metabolites, including cytotoxins have been studied [9–11]. The results of DNA typing methods have suggested a genomic polymorphism between *A. fumigatus* strains [12–16]. Nevertheless, the potential of a strain to invade tissues is still unclear and so is the question whether all *A. fumigatus* strains from the environment have the same probability of causing infection. Tang *et al.* [17] have suggested that certain isolates of *A. fumigatus* are

better adapted for growth in lung tissue. This hypothesis has been supported by a previous study of random amplified polymorphic DNA (RAPD) analysis of *A. fumigatus* strains from the environment and two distinct clinical entities – chronic aspergillosis without tissue invasion, and invasive aspergillosis. A 0.95-kb DNA fragment (EMBL/GenBank accession number: L35210) was correlated with the ability of *A. fumigatus* to invade tissues according to the immune status of the patient [18]. At present, no homologies have been found when this 0.95-kb fragment is compared with the whole known sequence banks, in particular with the nucleotide sequences of established virulence factors.

The previous models of invasive pulmonary aspergillosis (IPA) with cortisone-treated mice were developed to compare the virulence of a wild strain and its mutant. These required large inocula to establish a 100% mortality rate [5, 19, 20]. To highlight the diversity in virulence of different *A. fumigatus* strains independently of the immunological status of the host, an animal model of IPA with a low inoculum was developed. The difference in virulence of seven clinical strains from patients with non-invasive or invasive aspergillosis and four environmental strains was investigated and the results were compared with the presence or absence of the potential virulence marker.

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Materials and methods

Organisms

All *A. fumigatus* strains were isolated at the Grenoble University Hospital, France (Table 1). They were divided into three groups by accepted criteria for classification of aspergillosis [21]: non-invasive (nos 3, 5, 11 and 30), invasive (nos 13, 14 and 15) and environmental strains (E9, E10, E11 and E12). These strains have been deposited in the IHEM (Institute of Hygiene and Epidemiology Mycology, Brussels, Belgium) collection (Table 1). They were identified as *A. fumigatus* by their cultural characteristics and their microscopic features; all were stored for a short period on Sabouraud's dextrose agar at 4°C and for longer periods in glycerol 10% at -70°C.

Preparation of inocula

Each fungal strain was cultured on Sabouraud's dextrose agar for 72 h at 37°C. An inoculum was prepared by washing surface growth with Triton X100 0.1% in sterile physiological saline and filtering the resulting suspension through an 8-µm filter (Millipore). The conidia were concentrated by a succession of centrifugation steps and the cell number was determined in an haemocytometer [22].

Infection model

Swiss white female mice (OF1, IFAC REDO) weighing 26–28 g were immunosuppressed with hydrocortisone acetate (Lab. Roussel, Paris) 129 mg/kg by subcutaneous injections on day -4, -2, 0, +3 and +5. On day 0, groups of 5–10 mice were anaesthetised by inhalation with a mixture of absolute ethanol:chloroform:ether (1:2:3) and 4×10^5 *A. fumigatus* conidia in 30 µl of sterile saline were inoculated intranasally. Mice were kept in an air filter hood and received sterile food and bedding. After death, the heart, liver, brain and lungs were removed for mycological culture and PCR control. Concurrent control groups consisted of mice that were immunosuppressed and inoculated with 30 µl of Triton X100 0.1% in saline. The animals were observed twice a day for 30 days, after which inoculation and mortality were recorded.

DNA extraction

A sample of each isolate was inoculated into a 1.5-ml Eppendorf tube containing 500 µl of liquid Sabouraud medium and left to grow for 72 h 42°C. The mycelium was washed with sterile distilled water and crushed mechanically with a close fitting conidial grinder (Kontes, NJ, USA) in 300 µl of extraction buffer (200 mM Tris-HCl, pH 8.5, 500 mM NaCl, 25 mM EDTA, SDS 1%). The mixture was incubated at 55°C for 1 h with 1.5 µl of proteinase K (Sigma; 10 mg/ml) and extracted with phenol:chloroform. The aqueous phase was incubated with 15 µl of RNAase A

Table 1. Isolates of *A. fumigatus*, clinical information on each patient and virulence score in a murine model of IPA

Isolate no. (IHEM)	Patient sex	Infection/predisposing factors/outcome	Specimen	Inoculum (conidia)	Number of infected mice	Survival at 3 days (%)	Survival at 5 days (%)	Virulence score
0.95-kb-negative isolates								
3 (7954)	F	Pulmonary aspergilloma, sarcoidosis	Sputum	4×10^5	10	70	20	++
5 (7956)	M	Pulmonary aspergilloma	BAL	4×10^5	5	40	20	++
11 (7962)	F	Pulmonary aspergilloma, tuberculosis	BA	4×10^5	5	20	20	+++
15 (7966)	M	Brain aspergillosis, liver transplant, resolution	Brain biopsy	1.5×10^6	5	80	20	(+++)
E9		Wild environmental strain		4×10^5	5	80	60	+
E12		Wild environmental strain		4×10^5	10	50	40	+
0.95-kb-positive isolates								
13 (7964)	M	IPA, lymphoma, agranulocytosis, died (10 days)	BA	4×10^5	15	0	0	+++
14 (7965)	M	IPA, agranulocytosis, corticosteroid, died (20 days)	Tracheal aspirate	1.5×10^6	5	20	0	(+++)
30	M	Bronchial colonisation, lung transplant	BA	4×10^5	10	20	0	+++
E10		Wild environmental strain		4×10^5	5	20	20	+++
E11		Wild environmental strain		4×10^5	10	60	50	+

IHEM, Institute of Hygiene and Epidemiology Mycology (Brussels, Belgium); F, female; M, male; IPA, invasive pulmonary aspergillosis; BAL, bronchoalveolar lavage; BA, bronchial aspirate; +, ++, +++ and ++++ indicate the relative virulence score.

(Boehringer, 10 mg/ml) at 37°C for 1 h and extracted again with phenol:chloroform. After ethanol precipitation, the dried DNA was resuspended in 50 µl of TE buffer.

Amplification conditions

The reaction mixtures (30 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, Tween 20 0.005%, NP-40 0.005%, 100 µM each dNTP (Boehringer), 0.5 U of Replitherm^R DNA polymerase (Tebu), 0.1 µM of each primer and 50 ng of DNA template. Each reaction mixture was covered with 30 µl of mineral oil to prevent evaporation. Amplifications were carried out after an initial heat denaturation (95°C for 6 min) for 35 cycles, consisting of 20 s at 95°C, 30 s at 55°C and 60 s at 72°C. Amplification products were resolved by electrophoresis through agarose 1.5% gels.

Primers

Afp1 (TTGGGGAGATTACCGAACTGG) and Afp2 (CCTTGACAACCGTCCCATTTC) for the 0.95-kb fragment and two positive control primers Afc1 (GAGCGCCTGTATACTAAACCG) and Afc2 (GAGC GCCTTGATATGGATTAC) for a 1.4-kb fragment, deduced from the sequencing of fragments from RAPD patterns [18], were used.

Histopathology

The left and right lungs were dissected and fixed in formalin and embedded in paraffin. Sections were stained with Grocott's methenamine silver nitrate (GMS) and PAS for histological examination.

Statistical analysis

The time from inoculation to death was studied by survival analysis. The Kaplan-Meier method [23] was used to estimate the survival rates in a group (estimation for ungrouped data). Differences of survival rate between two groups were tested for statistical significance by the log-rank test procedure [24]. A *p* value ≤0.05 was considered statistically significant.

Results

Inoculum effect and reproducibility

Four clinical isolates (3, 13, 14 and 15) from different patients (Table 1) were tested in immunosuppressed mice by intranasal infection with different amounts of conidia: 5×10^6 , 1.5×10^6 and 4×10^5 conidia/mouse (Fig. 1A and B). A 100% mortality rate was observed at all of these concentrations. The survival rates after 3 days were 0% and 20% for mice inoculated with 5×10^6 conidia, compared with 20% and 80% with 1.5×10^6 conidia of the isolates 14 and 15, respectively (Fig. 1A). An inoculum effect was observed with

each strain (14: *p* < 0.02 and 15: *p* < 0.05). The inoculum variation between 1.5×10^6 and 4×10^5 conidia did not give significant differences (Fig. 1A and B) and enabled better reproducibility of the mortality curves at lower concentrations.

The infection experiments with 4×10^5 conidia of strains 3 and 13, were repeated two and three times, respectively. The survival curves of each strain showed good reproducibility (Fig. 1B). The control mice, immunosuppressed and inoculated with saline or inoculated with conidia in the absence of immunosuppression, did not develop any symptoms of IPA. The possibility of some conidia being taken up by the gastrointestinal tract was controlled by mycological cultures of the stomach. These were always negative. IPA was confirmed by histological sections of lung tissue from mice inoculated with 4×10^5 conidia of strain 13. The morphological appearance of fungal hyphae were characterised by long, thin, septate mycelia (4 µm in diameter) with dichotomous 45° branching (data not shown). Furthermore, the histological sections were similar to those observed in human IPA.

The difference in survival rate between the two strains (14 and 15) was not significant at higher conidia concentrations (5×10^6 and 1.5×10^6 conidia) but significant (*p* < 0.05) with strains 3 and 13 at lower concentrations (4×10^5 conidia). The level of inoculum chosen for all subsequent work was 4×10^5 conidia and two types of survival curves were observed: one showed a lower survival rate than the other. The different strains were typed by PCR with two pairs of primers: Afp1 and Afp2 amplified specifically the 0.95-kb fragment and Afc1 and Afc2, used in the same tube as a positive control, amplified a 1.4-kb fragment (Fig. 2).

Variation in virulence between strains

Nine strains were used to infect immunocompromised mice with 4×10^5 conidia: the survival rates after 3 days were relatively low with four of the strains (11, 13, 30, E10) and higher with the other five strains (3, 5, E9, E11, E12) (Table 1). When the animals inoculated with 4×10^5 conidia of the patient strains were considered as a whole, the survival rate of 25 mice infected with the 0.95-kb-positive strains was lower after 3 days than the survival rates of 20 mice infected with the 0.95-kb-negative strains (*p* < 0.02). The conidial concentration used with patient strains 14 and 15 (1.5×10^6 conidia) was high, but these two strains produced different survival rates after 3 days (20% and 80%, respectively) which suggested that strain 15 could be classed in the avirulent group and strain 14 in the virulent group (Table 1).

The survival rate after 3 days was higher for 30 mice inoculated with environmental strains than for 35 mice

Fig. 1. Mortality curves of infected mice. **a.** Δ controls; patient strain 14⁺, $\blacksquare$ 5×10^6 conidia, $\bullet$ 1.5×10^6 conidia; patient strain 15⁻, $\square$ 5×10^6 conidia, $\circ$ 1.5×10^6 (5 mice per group). **b.** Δ controls; $\triangle^{**}$ and $\square^{\S}$ patient strain 3⁻, 4×10^5 conidia; $\blacksquare^{\S}$, $\blacktriangle^{**}$ and $\bullet$ patient strain 13⁺, 4×10^5 conidia (5 mice per group). **c.** Δ controls (10 mice); $\blacksquare^*$ patient strain 30⁺, 4×10^5 conidia (5 mice); environmental strains E9⁻ ($\circ^{\S}$) and E10⁺ ($\bullet^{\S}$), 4×10^5 conidia (5 mice per group); environmental strains E11⁺ ($\blacktriangle^*$) and E12⁻ ($\square^*$), 4×10^5 conidia (10 mice per group). *Not significant; $^{\dagger}p < 0.2$; $^{\ddagger}p < 0.1$; $^{\S}p < 0.05$; $^{**}p < 0.02$.

inoculated with strains isolated from patients ($p < 0.001$). One strain (E10) out of four from the environmental group generated a rapidly fatal illness which was equivalent to the mortality curve of the 0.95-kb-positive strain 30 (Fig. 1C) but strain E10 was also 0.95-kb-positive (Fig. 2). The difference in survival rate after 3 days between the 0.95-kb-positive strains (E10 and 30) and the two 0.95-kb-negative strains (E9 and E12) was significant ($p < 0.05$) (Fig. 1C).

Discussion

The virulence of different *A. fumigatus* strains from different origins (clinical and environmental) was studied in a standardised in-vivo animal model of immunosuppression. Hydrocortisone acetate was chosen to suppress host defence mechanisms against *A. fumigatus* not only because of its profound effect on the alveolar macrophage conidiocidal activity [22] but also because of its action to increase the growth rate of *A. fumigatus* [25]. Cyclophosphamide, which induces neutropenia, was not used in addition to corticosteroids, although it has been used in other models of invasive aspergillosis [10]. The aim was not to suppress completely both lines of host defences, which would produce a deep immunosuppression and an overwhelming infection, but to compare different virulence levels of the strains.

Previous models of IPA with immunosuppression by corticosteroids have required large numbers of conidia (10^6 – 10^9 conidia/mouse) to establish a 100% mortality rate [4, 19, 20]. Tang *et al.* [6] and Smith *et al.* [9] decreased the number of conidia to achieve 100% mortality (8×10^5 conidia/mouse) by administration of the corticosteroid (112.5 mg/kg) in five stages. In the present study, the inoculum required to produce infection was optimised to give reproducible survival curves and a 100% mortality rate. The level of conidia was reduced to a lower limit (4×10^5 conidia) compared to previous models [6, 9]. The level of corticosteroid was slightly increased (129 mg/kg) and administered in five stages.

This model of IPA in mice provided different and reproducible mortality patterns with different strains. Previous results comparing virulence and genetic profiles have also suggested that virulence was not only the consequence of host immunity. RAPD patterns were used to look for a relationship between the genetic polymorphism of *A. fumigatus* strains and their ability to invade tissue. The virulence of 19 of the 24 strains was correlated with the presence or absence of the 0.95-kb fragment according to the immunological status and the infectious condition of each patient [18]. This procedure has already been used to identify genomic markers associated with the virulence genes of pathogenic fungi [26–28]. Further-

Fig. 2. PCR pattern generated with the genomic DNA of *A. fumigatus* strains: 3 (lanes 1 and 2), 13 (3 and 4), 14 (5 and 6), 15 (7 and 8), E9 (9 and 10), E10 (11 and 12) with primers Afp1 and Afp2 (lanes 1, 3, 5, 7, 9, 11) and the two primer pairs: Afp1, Afp2, and Afc1, Afc2 (lanes 2, 4, 6, 8, 10). Lane 13 shows amplification without DNA. Lane M: 1-kb DNA ladder marker graded in kb.

more, the virulence factors of *A. fumigatus* have still not been elucidated and this original approach may highlight a virulence determinant without prior knowledge of the DNA sequence.

This IPA infection model allowed a comparison of the virulence of 11 strains with the presence or the absence of a molecular marker. The 0.95-kb-positive strains gave a lower survival rate than the 0.95-kb-negative strains. Furthermore, strains 15 and 30 were isolated from invasive and non-invasive aspergillosis, but by PCR were assigned as 0.95-kb-negative and -positive strains, respectively. With regard to strain 15, although the dissemination of the infection involved the brain, the patient responded to treatment, despite a poor prognosis. This may suggest that the strain was only weakly virulent. Strain 30 was isolated from bronchial colonisation and the immune status of the host could not be evaluated. In the IPA model, the virulence of the 0.95-kb-negative strain (15) was weaker than that of the 0.95-kb-positive strain (30). This result demonstrates a correlation between the 0.95-kb fragment and virulence, irrespective of the origin of the strain (non-invasive or invasive aspergillosis).

The environmental strains showed weaker virulence potential than the patient isolates. Nevertheless, these data suggest that certain strains have acquired specific virulence determinants, which are generally thought to evolve in response to selective pressure imposed by growth in animals. Epidemiological investigations by moderately repetitive sequences and RAPD have shown that patients with invasive aspergillosis were infected by only one or two strain types, although a wide variety of isolate types exist in their environment [14, 15, 17]. This epidemiological approach to virulence will allow a consideration of other factors by

studying the difference in genomic expression between strains, which can be distinguished by several parameters: sample origin (patients with non-invasive or invasive aspergillosis and the environment), molecular markers [14, 18] and the degree of virulence in the animal model of IPA described here.

The infection profile and the molecular marker showed a correlation whatever the origin of the strains, but this correlation was not absolute. In other reports, a comparison in virulence between wild-type and mutant strains with one or two inactivated genes in immunosuppressed mice has shown that these proteins were not essential to prevent the fungus from colonising the lung tissues of mice [5–9]. Virulence is probably multifactorial and due to several enzymatic proteins or toxins acting either sequentially or simultaneously. A 0.95-kb fragment of genomic DNA alone could not be responsible for the total pathogenic potential of the fungus. However, the results suggest that it is strongly associated with the ability to invade lung tissues.

The animal model of IPA with a low inoculum has allowed confirmation of the hypothesis that pathogenicity not only depended on host immunity but also on fungus-related factors. Furthermore, the differential capacity of *A. fumigatus* strains to invade mice tissues is correlated to the molecular marker and confirms previous data [18]. Therefore, we intend to screen a cDNA bank to discover whether this fragment corresponds to a gene characteristic of virulence or whether it is an untranslated sequence linked to growth ability.

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