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Virulence of *Aspergillus fumigatus* strains investigated by random amplified polymorphic DNA analysis

P. MONDON, J. THÉLU, B. LEBEAU, P. AMBROISE-THOMAS and R. GRILLOT

Département de Parasitologie-Mycologie Médicale et Moléculaire (DP3M), CNRS EP J0078, Faculté de Médecine, Université Joseph Fourier Grenoble I, Domaine de La Merci 38706 La Tronche, France

Summary. A possible relationship between the ability of *Aspergillus fumigatus* strains to invade tissues and genetic polymorphism was studied by random amplified polymorphic DNA (RAPD) analysis. One hundred randomly designed oligonucleotide decamers were examined with DNA of three reference strains, eight environmental isolates and 21 isolates from two distinct clinical situations: non-invasive aspergillosis (predominantly aspergilloma) and invasive aspergillosis. One primer (OPQ 6) was found to generate a reproducible amplification product that enabled distinction between the two groups according to the presence or absence of a 0.95-kb fragment that correlated with the nature of the infection (non-invasive or invasive) and immune status of the patient. The results indicated that the pathogenicity of *A. fumigatus* was related not only to the host's immune status but also to the virulence of the strain of *A. fumigatus*.

Introduction

The genus *Aspergillus* is one of the most ubiquitous micro-organisms worldwide. Of particular importance in medicine is *A. fumigatus*, an opportunist pathogen responsible for several types of respiratory diseases. The extent of disease depends on the host's immune defences; in immunocompromised hosts, it may cause fatal invasive aspergillosis. However, immunosuppression cannot explain all the pathological features of aspergillosis. Several products of *A. fumigatus* (proteases, catalase, cytotoxin) have been suggested as determinants of pathogenicity, although their role is still unclear.^{1–4} Epidemiological investigation and control of these mycoses require reproducible methods to distinguish strains and establish whether some strains are particularly associated with the various clinical types of aspergillosis. The conventional typing methods (morphotyping, serotyping) use phenotypic characteristics that depend on environmental factors and are considered to be too variable to be conclusive. Such problems may be resolved by employing DNA-based techniques of molecular biology.

In recent years, molecular typing methods have been employed for restriction endonuclease analysis of genomic DNA (e.g., RFLP).^{5,6} Data from these

methods suggest a genomic polymorphism between *A. fumigatus* strains. However, such methods are both time-consuming and labour-intensive, and require preliminary in-vitro multiplication that can modify the patterns of the strain. To avoid these problems, the random amplified polymorphic DNA (RAPD) technique⁷ has been developed. This technique employs single short primers (decamers) with arbitrary nucleotide sequences in a polymerase chain reaction (PCR) to amplify genomic DNA randomly. The system has already generated banding patterns that correlate with the pathogenicity of two fungal plant pathogens, *Leptosphaeria maculans*⁸ and *Fusarium solani*.⁹

The purpose of this study was to investigate the DNA of three reference strains, eight environmental isolates and 21 isolates of *A. fumigatus* from two distinct clinical situations—chronic aspergillosis without tissue invasion (12 cases) and invasive aspergillosis (nine cases)—to identify a relationship between the genetic polymorphism of these isolates and their ability to invade tissues.

Materials and methods

Organisms

A. fumigatus isolates were divided into three groups (tables I and II) by accepted criteria of aspergillosis classification.¹⁰ Group I consisted of one reference

strain (Longbottom) and 12 *A. fumigatus* isolates from non-invasive (and non-allergic) aspergillosis isolated from immunologically normal hosts or patients with only modest impairment of defence mechanisms. Six isolates were from patients with pulmonary aspergilloma (nos. 2, 3, 5, 8, 10 and 11), one from pleural aspergilloma (no. 6), three from fungus balls of the maxillary sinus (nos. 1, 7 and 12) and two from bronchial colonisation following corticosteroid therapy but without invasion (nos. 4 and 9). Group 2 comprised two reference strains (CBS 144-89 and its $\Delta 18$ mutant) and nine isolates from proven or highly probable invasive aspergillosis in patients with acute leukaemia, malignant lymphoma or following organ transplantation; three were from invasive pulmonary aspergillosis (nos. 13, 14 and 20), two from disseminated aspergillosis involving lung and other organs (nos. 15 and 21), one from osteomyelitis (no. 16), two from invading infections originating in the sinus (nos. 17 and 18), and one from acute necrotising broncho-pneumonia (no. 19). The following clinical information was obtained from each patient: infection type, site, predisposing factors, therapy and outcome. Group 3 consisted of eight saprophytic isolates (table II) from environmental samples collected in Grenoble hospitals (E1-E8). All clinical samples were obtained from the Grenoble University Hospital, France, and strains CBS 144-89 and $\Delta 18$ were kindly provided by M. Monod (Laboratory of Mycology, Lausanne, Switzerland).³ Isolates were identified as *A. fumigatus* by their cultural characteristics and microscopic features, and were stored on Sabouraud's dextrose agar at 4°C and in glycerol broth (10%) at -70°C.

Preparation of DNA

DNA was extracted by a rapid small-scale method adapted from that of Cenis.¹¹ Briefly, each isolate was subcultured overnight on Sabouraud's dextrose agar at 37°C. One colony was inoculated into a 1.5-ml Eppendorf tube in liquid Sabouraud medium (500 µl) and incubated for 72 h at 25°C. The mycelium was pelleted by centrifugation at 13000 *g* for 5 min and washed with 500 µl of TE buffer (200 mM Tris-HCl, pH 8.5, 1 mM EDTA). The TE was decanted and 300 µl of extraction buffer (200 mM Tris-HCl, pH 8.5, 500 mM NaCl, 25 mM EDTA, SDS 1%) was added. The mycelium was crushed with a close fitting conidial grinder (Kontes, NJ, USA) at 200 rpm. Sodium acetate (150 µl; 3 M, pH 5.2) was added and tubes were placed at -20°C for 10 min. Extracts were centrifuged and the supernates were collected. An equal volume of isopropanol was added and extracts were incubated for 5 min at room temperature, then centrifuged at 13000 *g* for 20 min. The pellet was washed with ethanol 70%, vacuum dried for 5 min and resuspended in TE (50 µl) containing RNAase A (Boehringer) 3 µg/ml.

Amplification conditions

The reaction mixtures (30 µl) contained 20 mM Tris-HCl (pH 8.3), 200 mM KCl, 1.5 mM MgCl₂, Tween 20 0.1%, NP-40 0.1%, 75 µM each dNTP (Boehringer), 0.5 U of Replitherm® DNA polymerase (Epicentre), and 0.2 µM of arbitrary primer or 0.1 µM of each specifically designed primer. The DNA template used contained 50 ng and 10 ng for arbitrary or specific primers, respectively. Each reaction mixture was overlaid with mineral oil (30 µl) to prevent evaporation. Amplifications were performed after initial heat denaturation (94°C for 6 min). For arbitrary short primers, 40 cycles of 5 s at 94°C, 30 s at 37°C and 60 s at 63°C were used; for specific long primers, 30 cycles of 20 s at 94°C, 30 s at 55°C and 60 s at 72°C were used. Amplification products were resolved by electrophoresis on agarose 1.5% gels and stained with ethidium bromide 0.5 µg/ml.

RAPD primers

One hundred RAPD primers designed according to the following theoretical parameters—G-C content > 60%, no dyad symmetry and non-self complementary—were purchased from Operon Technologies Inc., Alameda, CA, USA.

DNA cloning and sequencing

The DNA template was amplified with a short primer and 2 µCi [α^{35} S] dATP (Amersham). Ten µl of each reaction mixture were separated by electrophoresis on an acrylamide 6%, urea 50% sequencing gel and subjected to autoradiography. Bands of interest were collected, the gel was homogenised with a

Fig. 1. RAPD fragments generated with primer OPQ 6 and genomic DNA of *A. fumigatus* isolates. a: (lanes 1-13) non-invasive infections (Longbottom and isolates 1-12); b: (lanes 1-11) invasive infections (isolates 13-21, CBS 144-89 and $\Delta 18$). PCR products generated with primers Afp1 and Afp2 and genomic DNA of isolates 7 (A: 14) and 13, 14, 18, 19, 20, CBS 144-89, $\Delta 18$ (B: lanes 12-18). Lane M: 1-kb DNA ladder marker (Gibco-BRL).

Table 1. Isolates of *A. fumigatus*, clinical information on each patient and amplification results

Isolate no.	Patient sex	Infection/predisposing factors/outcome	Sample	Presence of 0.95-kb band
Group 1: non-invasive aspergillosis				
Longbottom		Aspergilloma wild-type strain	...	—
1	F	Maxillary sinus aspergilloma	Surgical removal	—
2	M	Pulmonary aspergilloma	BA	—
3	F	Pulmonary aspergilloma, sarcoidose	Sputum	—
4	M	Bronchial colonisation, corticosteroid therapy	BAL	—
5	N	Pulmonary aspergilloma	BAL	—
6	M	Pleural aspergilloma, following lung carcinoma	Surgical removal	—
7	M	Maxillary sinus aspergilloma, dental procedure	Surgical removal	+
8	F	Pulmonary aspergilloma, tuberculosis, bronchiectasis	Sputum	—
9	M	Bronchial colonisation, corticosteroid therapy	BA	—
10	F	Pulmonary aspergilloma, tuberculosis	BA	—
11	F	Pulmonary aspergilloma, tuberculosis	BA	—
12	F	Maxillary sinus aspergilloma	Sinus drainage	—
Group 2: invasive aspergillosis				
13	M	IPA, lymphoma, agranulocytosis, died (10 days)	BA	+
14	M	IPA, agranulocytosis, corticosteroid, died (20 days)	Tracheal aspirate	+
15	M	Brain A, liver transplant, resolution	Brain biopsy	—
16	M	Vertebral A, AML, neutropenia, resolution	Vertebral biopsy	—
17	M	Orofacial A, ALL, agranulocytosis, resolution	Tissue biopsy	—
18	M	IA, sinusitis, ALL, died (105 days)	Sinus drainage	+
19	M	Necrotising PA, Hodgkin, radiation fibrosis, extension	Sputum	+
20	F	IPA, lung transplant, favourable outcome	BA	+
21	M	IA, ALL, agranulocytosis, resolution	Sputum	—
CBS 144-89		IA, wild-type virulent strain	...	+
Δ 18		IA, mutant strain derived from CBS 144-89	...	+

conical grinder and 10 μ l of the mixture were amplified directly as described previously, without further DNA purification. The amplification products were cloned with a TA cloning kit (Invitrogen). The insert DNA was sequenced by the termination method of Sanger *et al.*,¹² with the Sequenase 2.0 kit (United States Biochemical Corp.) and [α^{35} S] dATP (Amersham). On the basis of the sequence, the oligonucleotide primer sequences were selected with the Mac Vector program (IBI), and synthesised by Eurogentec (Belgium).

Results

Reproducible amplifications were obtained with 40% of the primers. The number of bands varied between five and 10 with size range of 0.3–2.5 kb. The patterns obtained with the majority of primers showed no difference between *A. fumigatus* strains, although they allowed the differentiation of *A. fumigatus* from other *Aspergillus* spp. (data not shown). However, the OPQ 6 primer (GAGCGCCTTG) generated RAPD products that enabled differentiation among the *A. fumigatus* groups.

The 24 clinical isolates (reference strains included) of *A. fumigatus* amplified with OPQ 6 primer revealed four types of profiles (fig. 1). The banding pattern showed seven major bands, five of which were conserved (1.4, 1.0, 0.8, 0.7 and 0.65 kb), and two of which were variably present (1.2 and 0.95 kb). Together,

these last two allowed the discrimination of different profiles but did not distinguish between invasive and non-invasive groups. However, when only the 0.95-kb fragment was considered, there was some correlation between profile types and clinical types of aspergillosis (table 1). The presence or absence of this fragment allowed the separation of strains into two distinct groups. Group 1 (fig. 1A) comprised isolates from non-invasive infections that provided profile patterns without this fragment, with the exception of isolate no. 7 (a strain isolated from a maxillary sinus aspergilloma). Group 2 (fig. 1B) comprised isolates from invasive infections that showed profiles with a band at 0.95 kb in five of nine isolates and in the reference strains CBS 144-89 and Δ 18. The band was absent from the invasive strains 15, 16, 17 and 21. The RAPD pattern was stable within a single strain over many generations and between different colonies originating from a single isolate (data not shown). The cloning and sequencing of the 0.95-kb fragment (EMBL/GenBank data base accession number: L35210) has allowed the design of more specific primers, Afp1 (TTGGGGA-GATTACCGAACTGG) and Afp2 (CCTTGACAA-CCGTCCCATTTC). These primers confirmed the presence or absence of this band (fig. 1) with greater sensitivity (quantity of DNA used in PCR was 10-fold less). Primers Afc1 (GAGCGCCTTGATATACTAA-ACCG) and Afc2 (GAGCGCCTTGATATGGA-TTAC) were deduced from the sequencing of a conserved fragment (1.4 kb) from the RAPD pattern. These primers were used in the same reaction with Afp1 and Afp2 as positive controls. This system was

Table II. Details of environmental isolates (Group 3)

Isolate no.	Location (date of isolation)	Presence of 0.95-kb band
E1	Haematology unit, above the cupboard (22.12.93)	+
E2	Haematology unit, above the serviette box (22.12.93)	+
E3	Haematology unit, cupboard with wheels (22.12.93)	—
E4	Haematology unit, instrument cupboard (22.12.93)	—
E5	Dwelling, bedroom, head-board (27.01.94)	+
E6	Dwelling, bedroom, wardrobe (27.01.94)	—
E7	Dwelling, bedroom, window (27.01.94)	—
E8	Dwelling, bedroom, light switch (27.01.94)	—

Fig. 2. Results of PCR amplification of DNA isolated from environmental strains E1 (lanes 1, 2), E2 (3, 4), E3 (5, 6), E4 (7, 8), E5 (9, 10), E6 (11, 12), E7 (13, 14) and E8 (15, 16) with primers Afp1 and Afp2 (lanes 1, 3, 5, 7, 9, 11, 13, 15) and primers Afp1, Afp2, Afc1 and Afc2 (lanes 2, 4, 6, 8, 10, 12, 14, 16). Lane M: 1-kb DNA ladder marker (Gibco-BRL).

used to amplify the DNA of eight environmental strains (table II) and the 0.95-kb fragment was present in three of eight isolates (E1, E2, E5) (fig. 2).

Discussion

Molecular studies with fingerprinting techniques have been used to type *A. fumigatus* isolates for epidemiological studies of nosocomial aspergillosis.¹³⁻¹⁶ The aim of this study was not to distinguish isolates but to identify a RAPD marker that correlated with the capacity to cause disease. The role of virulence factors in invasive aspergillosis has been studied.¹⁷ Although these virulence determinants are not fully understood, the results suggest their importance in pathogenicity.¹⁻⁴ The RAPD technique was chosen because it enabled the study of the genome of *A. fumigatus* in the absence of sequence information.

The results showed that the presence of the 0.95-kb fragment correlated with invasive disease in 19 (80%) of 24 patient isolates. However, *A. fumigatus* is an opportunist pathogen and the disease evolution depends on two factors—host and fungus. The relationships between a probable pathogenic marker and the potential virulence of strains has to be correlated with two parameters, the immunological status of the host and the severity of infection. Both the reference CBS 144-89 strain and its mutant $\Delta 18$ contained the 0.95-kb band. The $\Delta 18$ mutant was constructed by disruption of the gene encoding the alkaline protease, a highly probably virulence factor, but no significant differences in mortality were reported when these strains were compared in murine models of invasive pulmonary aspergillosis.² It is likely that virulence factors act in combination and, when considered singly, alkaline protease has only a minor effect in virulence. There was no homology when the 0.95-kb fragment was compared with the sequence of established virulence factors, in particular the alkaline proteases.

The isolates nos. 13, 14, 18, 19 and 20 from invasive infections (table I) provided profile patterns containing the 0.95-kb band. Four of five patients failed to respond to early and aggressive antifungal therapy. Thus, outcome may be related to virulence of fungal strains. The patient with isolate no. 20 had a favourable outcome but was less immunosuppressed than the other patients in the group. Some exceptions were found. One of 13 non-invasive isolates (no. 7) contained the 0.95-kb band although there were no features of invasive disease. Conversely, the 0.95-kb band was absent in strains that generated invasive disease in four cases (nos. 15, 16, 17 and 21). However, these patients responded to treatment, despite a poor prognosis as determined by either site of infection (brain: no. 15), or the degree of immunosuppression (severe aplasia: nos. 16, 17 and 21). It is possible that the favourable outcome was due to weaker virulence of the strains. Tang *et al.*¹⁸ have studied isolates from patients and the environment with RAPD and their findings suggest that certain isolates are better adapted for growth in lung tissue. The amplification pattern of environmental strains showed a variable presence of the 0.95-kb band regardless of geographical origin.

These results suggest that the virulence of *A. fumigatus* is variable from strain to strain and related to a genetic polymorphism. The pathogenicity seems to depend on not only host immunity but also fungus-related factors. It is not known whether all *A. fumigatus* strains from the environment have the same capacity to invade human tissues or whether the risk of developing invasive infection in immunosuppressed patients is higher with strains with a modified genome, possibly correlated to our molecular marker. Studies

with more patients and environmental isolates and investigation of the pathogenicity of clinical and environmental isolates in a murine model of invasive pulmonary aspergillosis are needed to investigate this further.

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References

- Kolattukudy PE, Lee JD, Rogers LM, Zimmerman P *et al.* Evidence for possible involvement of an elastolytic serine protease in aspergillosis. *Infect Immun* 1993; **61**: 2357–2368.
- Monod M, Paris S, Sarfati J, Jatton-Ogay K, Ave P, Latgé J-P. Virulence of alkaline protease-deficient mutants of *Aspergillus fumigatus*. *FEMS Microbiol Lett* 1993; **106**: 39–46.
- Markaryan A, Morozova I, Yu H, Kolattukudy PE. Purification and characterization of an elastolytic metalloprotease from *Aspergillus fumigatus* and immunoelectron microscopic evidence of secretion of this enzyme by the fungus invading the murine lung. *Infect Immun* 1994; **62**: 2149–2157.
- Smith JM, Davies JE, Holden DW. Construction and pathogenicity of *Aspergillus fumigatus* mutants that do not produce the ribotoxin restrictocin. *Mol Microbiol* 1993; **9**: 1071–1077.
- Burnie JP, Coke A, Matthews RC. Restriction endonuclease analysis of *Aspergillus fumigatus* DNA. *J Clin Pathol* 1992; **45**: 324–327.
- Denning DW, Clemons KL, Hanson LH, Stevens DA. Restriction endonuclease analysis of total cellular DNA of *Aspergillus fumigatus* isolates of geographically and epidemiologically diverse origin. *J Infect Dis* 1990; **162**: 1151–1158.
- Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 1990; **18**: 6531–6535.
- Goodwin PH, Annis SL. Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. *Appl Environ Microbiol* 1991; **57**: 2482–2486.
- Crowhurst RN, Hawthorne BT, Rikkerink EHA, Templeton MD. Differentiation of *Fusarium solani* f. sp. *cucurbitae* races 1 and 2 by random amplification of polymorphic DNA. *Curr Genet* 1991; **20**: 391–396.
- Bodey GP, Vartivarian S. Aspergillosis. *Eur J Microbiol Infect Dis* 1989; **8**: 413–437.
- Cenis JL. Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Res* 1992; **20**: 2380.
- Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 1977; **74**: 5463–5467.
- Aufaure-Brown A, Cohen J, Holden DW. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. *J Clin Microbiol* 1992; **30**: 2991–2993.
- Van Belkum A, Quint WGV, De Pauw BE, Melchers WJG, Meis JF. Typing of *Aspergillus* species and *Aspergillus fumigatus* isolates by interrepeat polymerase chain reaction. *J Clin Microbiol* 1993; **31**: 2502–2505.
- Loudon KW, Burnie JP, Coke AP, Matthews RC. Application of polymerase chain reaction to fingerprinting *Aspergillus fumigatus* by random amplification of polymorphic DNA. *J Clin Microbiol* 1993; **31**: 1117–1121.
- Girardin H, Sarfati J, Traoré F, Dupouy-Carnet J, Derouin F, Latgé JP. Molecular epidemiology of nosocomial invasive aspergillosis. *J Clin Microbiol* 1994; **32**: 684–690.
- Denning DW. Epidemiology and pathogenesis of systemic fungal infections in the immunocompromised host. *J Antimicrob Chemother* 1991; **28** Suppl B: 1–16.
- Tang CM, Cohen J, Rees AJ, Holden DW. Molecular epidemiological study of invasive pulmonary aspergillosis in renal transplantation unit. *Eur J Microbiol Infect Dis* 1994; **13**: 318–321.