JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1996, p. 2595–2597 Vol. 34, No. 10
0095-1137/96/$04.00+0

Genotypic Characterization of Sequential *Aspergillus fumigatus* Isolates from Patients with Cystic Fibrosis

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Received 30 April 1996/Returned for modification 12 June 1996/Accepted 12 July 1996

Twenty-three sequential *Aspergillus fumigatus* sputum isolates, which had been collected over a period of 2 years, from two patients with cystic fibrosis were genotyped by random amplified polymorphic DNA PCR and restriction fragment length polymorphism analysis. In patient B, one genotype was predominantly present in the sputum samples, while in the other patient up to nine different genotypes were identified. This study suggests that different patterns of colonization with *A. fumigatus* exist in patients with cystic fibrosis.

Cystic fibrosis (CF) is an ultimately lethal, autosomal, recessive hereditary disease involving dysfunction of the exocrine glands. The lungs are a major target of disease, and the presence of thick tenacious secretions favors growth of certain microorganisms, e.g., *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Furthermore, in 10 to 57% of CF patients the respiratory tract is also colonized by *Aspergillus* species (3, 15, 18). *Aspergillus fumigatus* is the fungus most commonly isolated, and this fungus was found to be the next most persistent microorganism in sputum secretions after *P. aeruginosa* (3). Sensitization to *Aspergillus* allergens may lead to the development of allergic bronchopulmonary aspergillosis in up to 11% of patients (13–15). However, although *A. fumigatus* can be cultured from sequential sputum samples obtained from CF patients, it is not known whether colonization of the respiratory tract with *A. fumigatus* is due to one or several strains or that frequent recolonization takes place. In the present study, we have typed *A. fumigatus* isolates cultured from sputum samples of two patients with CF by use of restriction fragment length polymorphism (RFLP) analysis and PCR amplification of random polymorphic DNA fragments (RAPD-PCR). These molecular typing methods have previously been used with other patient groups to genotype *A. fumigatus* isolates (1, 2, 4–6, 10, 11, 19, 21).

Sputum samples obtained from CF patients were routinely cultured on Sabouraud glucose 2% agar for 4 days at 29°C. *A. fumigatus* was identified by cultural characteristics and the morphology of conidiophores and conidia. Mycelial growth of one colony was peeled off, suspended in 1 ml of glycerol-peptone broth, and stored at −80°C until testing. Twelve and 11 *A. fumigatus* isolates cultured from sequential sputum samples from patients A and B, respectively, were available for molecular typing, and each test was performed in duplicate. The *Aspergillus* isolates were defrosted, plated onto Sabouraud agar, and incubated at 42°C. Conidia from each isolate were inoculated into 5 ml of Sabouraud liquid medium and incubated on a rotary shaker (24 h, 37°C, 200 rpm). Mycelia were harvested by centrifugation for 10 min at 5,000 × *g*. Complete cell destruction was achieved with a mortar under liquid nitrogen (7). One milliliter of extraction buffer (2% [wt/vol] hexadecyltrimethylammonium [Sigma Aldrich, Axel, The Netherlands], 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA) was added, and the mixtures were incubated at 55°C for 30 min. Next, the samples were centrifuged for 5 min at 13,000 × *g* (Eppendorf centrifuge). The supernatant was collected, and nucleic acids were purified by phenol-chloroform-isoamyl alcohol (25:24:1) extraction followed by precipitation with ethanol for 1 h at −80°C and washed twice with 80% ethanol (12). Finally, the samples were centrifuged for 1 min at 13,000 × *g* and dissolved in 200 μl of distilled water. RAPD-PCR fingerprinting was performed as described previously by using primer R108 (5′-GTATTGCCCT) (2). Amplifications were carried out by using a 40-cycle program with each cycle consisting of denaturation for 1 min at 94°C, annealing for 1 min at 35°C, and elongation for 1 min at 72°C (21). Amplification products (10 μl) were separated by electrophoresis through 1.5% agarose gels containing ethidium bromide. Gels were photographed and interpreted by visual examination. Both faint and intense bands were included in the interpretation of the pattern, and patterns differing by more than one band were categorized as different genotypes. RFLP fingerprinting was performed exactly as described previously (4). Briefly, DNA from each *A. fumigatus* isolate was digested with 4 U of *Eco*RI per μg of DNA at 37°C for 16 h. DNA fragments were separated on a 0.7% agarose gel (1 μg of DNA per lane; 16 h at 25 V) and transferred to a nylon filter (Hybond N^+; Amersham) with a vacuum transfer filter. A repetitive 6,914-bp DNA probe (16) was labelled by the random priming method (Megaprime; Amersham). Prehybridization and hybridization were performed at 65°C in a solution of 5× SSPE (1× SSPE contains 10 mM sodium phosphate [pH 7.5], 10 mM EDTA, and 0.18 M NaCl), 5% dextran sulfate, 150 μg of sheared denatured salmon sperm DNA per ml, and 0.3% sodium dodecyl sulfate (SDS). Filters were washed with a solution of 2× SSPE–0.3% SDS at 65°C and then exposed to Kodak X-Omat AR film with an intensifying screen for 1 to 3 h at 80°C. The RFLP banding patterns were interpreted by visual inspection, and a single-band discrepancy between isolates was considered to indicate a different genotype. Medical records of both patients were reviewed for clinical signs and symptoms of allergic bronchopulmonary aspergillosis and the use of corticosteroids or antifungal agents.

Patient A was 19 years old, and besides CF he also suffered

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TABLE 1. Dates of culture, diagnostic yields, and results of genotyping by RAPD and RFLP of *A. fumigatus* isolates from sputum samples obtained from two patients with CF

Patient and date of culture (day/mo/yr)	Diagnostic yield[a]	Isolate	Genotype	
			RAPD	RFLP
A				
24/12/91	2	1	A	1
24/12/91	2	2	A	1
20/10/92[b]	1	3	B	2
09/03/93[b]	1	4	C	3
22/09/93[b]		NA[c]		
25/11/93[b]		NA		
29/11/93[b]	2	5	D	4
02/12/93[b]	1	6	D	4
28/01/94	1	7	E	5
28/01/94	1	8	E	6
31/01/94	1	9	A	7
04/02/94	2	10	F	8
07/02/94		NA		
10/02/94	1	11	G	9
24/03/94	3	12	A	7
B				
23/03/92	1	13	H	10
26/03/92	1	14	I	4
30/03/92	1	15	H	4
01/06/92	1	16	H	10
15/06/92	1	17	H	10
25/01/93	1	18	H	4
28/01/93	1	19	H	10
14/05/93	1	20	H	10
12/08/93	1	21	H	10
06/10/93	1	22	J	11
18/02/94	1	23	H	10

[a] 1, 1 or 2 colonies; 2, 2 to 5 colonies; 3, 5 to 10 colonies.
[b] Sample obtained during treatment with itraconazole.
[c] NA, isolate not available for fingerprinting.

from insulin-dependent diabetes mellitus and liver cirrhosis complicated by esophageal varices. Culture of 54 consecutive sputum samples, which had been collected over a 25-month period, yielded *A. fumigatus* in 13 samples, and 12 isolates were available for genotyping (Table 1). During this period the patient was treated with antibacterial agents for recurrent episodes of bacterial pneumonia. Because *A. fumigatus* was cultured from the sputum and precipitins to *A. fumigatus* were detected in the serum, the patient was treated with itraconazole (200 mg three times a day) between June and October 1992, between January and November 1993, and during 10 days in December 1993.

CF was diagnosed in patient B at an age of 2.5 years, and *A. fumigatus* was cultured from sputum samples for the first time when she was 7 years of age (29 December 1989). Between March 1992 and February 1994, 12 of 41 consecutive cultures of sputum samples yielded *A. fumigatus*, and 11 isolates were available for genotyping (Table 1). During this period the patient was treated with antibacterial agents for recurrent episodes of bacterial pneumonia. There was no evidence of allergic bronchopulmonary aspergillosis, and specific immunoglobulin E antibodies to *A. fumigatus* were not detected by the *Aspergillus* radioallergosorbent test. Furthermore, the patient had not been treated with antifungal agents or corticosteroids. For both patients, sputum samples were collected regularly at each visit to the outpatient clinic and during admissions, and there was no evidence of the presence of cavitary lung lesions.

RAPD and RFLP banding patterns of the 23 *A. fumigatus* isolates are shown in Fig. 1 and 2, respectively, and are summarized in Table 1. Both RAPD and RFLP discriminated three genotypes in patient B, of which one (RAPD genotype H, RFLP genotype 10 [Table 1]) was predominantly present in the sputum samples obtained during a 23-month period. However, for patient A, eight and nine genotypes were detected by RAPD and RFLP, respectively. This patient had received long-term treatment with antifungal agents which did not result in eradication of the fungus from the respiratory tract. The contribution of antifungal treatment to the large number of colonizing genotypes remains unclear, although genotypic unrelated strains remained present in the sputum after discontinuation of itraconazole therapy (Table 1). Other factors such as the level of exposition to environmental spores may also be important (17), since genotyping of environmental *A. fumigatus* isolates shows that great genomic variation may exist (6). Little is known about the pattern of colonization in CF patients, but genotyping of *A. fumigatus* isolates from non-CF patients with invasive aspergillosis (6, 19) and aspergil-

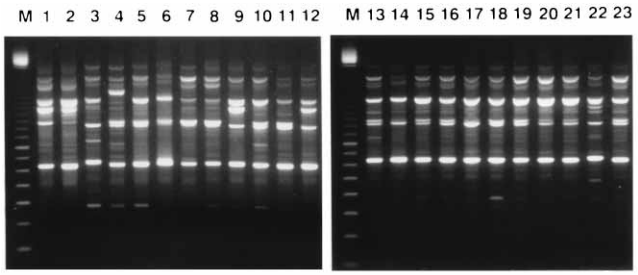

FIG. 1. RAPD patterns of *A. fumigatus* DNA samples with primer R108. Lanes 1 to 12, patient A; lanes 13 to 23, patient B; lane M, marker. Lane numbers correspond to isolate numbers in Table 1.

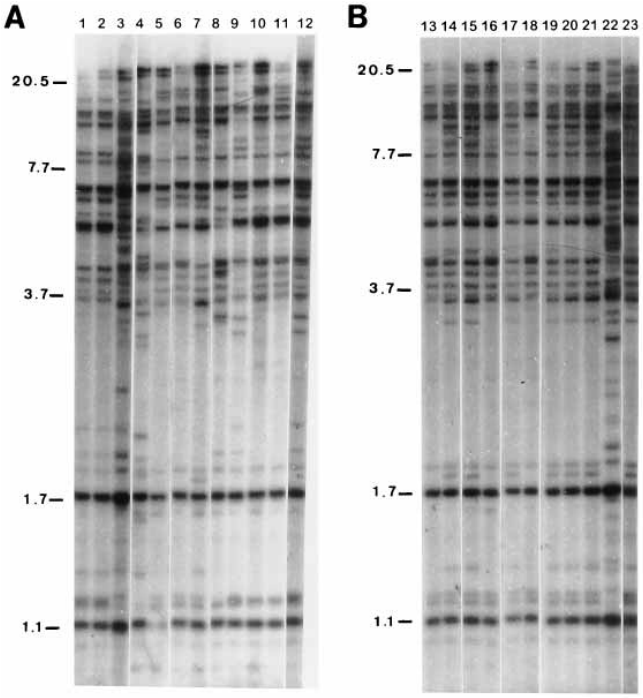

FIG. 2. RFLP with *Eco*RI-digested *A. fumigatus* DNA samples. Lanes 1 to 12, patient A; lanes 13 to 23, patient B. Lane numbers correspond to isolate numbers in Table 1.

loma (5) strongly suggests that these patients are colonized and infected by a single and unique strain. The patterns of *Aspergillus* colonization in CF patients resemble that of *P. aeruginosa* colonization. Molecular typing of *P. aeruginosa* isolates from sputum samples of CF patients revealed that some patients are colonized with a rather constant *Pseudomonas* flora while in others the composition of the flora may fluctuate considerably (8, 9).

The highest degree of discriminatory power of RAPD genotyping with *A. fumigatus* isolates has been achieved with primer R108 (2, 10). The RAPD method is easy to perform and allows large numbers of isolates to be analyzed within a short period of time. However, the discriminatory power of primer R108 alone may not be sufficient for epidemiological study of isolates from individual patients, and therefore the results of RAPD were compared with those of RFLP, which has been shown to provide a high level of discrimination (1, 4). In the present study, the results of RAPD fingerprinting and RFLP analysis were not always in agreement. RAPD pattern A was found for RFLP types 1 and 7, RAPD pattern E corresponded to RFLP types 5 and 6, and RAPD pattern H corresponded to RFLP types 4 and 10 (Table 1). Furthermore, RAPD patterns D and H corresponded to RFLP type 4. Both RAPD and RFLP analysis were repeated (data not shown), but although minor variations in band intensity were observed, the overall banding pattern and interpretation were consistent. Previous comparisons between R108-primed RAPD and RFLP typing methods have shown both full agreement (19) and discordance (1, 11). Factors such as buffer composition, primer concentration, thermal cycler performance, and gel electrophoresis time may play an important role in the band pattern and affect the discriminatory power of the primer. These factors may also limit the utility of comparison of results from different research centers (1). Interlaboratory variation of RAPD genotyping has been studied for *S. aureus* (20). Despite an effort by the authors to control several variables by standardization of DNA extraction, primer quality, and PCR protocol, major differences among laboratories were encountered. Although the majority of epidemiologically related *S. aureus* strains were clustered correctly by the different institutes, the resolution of PCR fingerprinting varied from 50 to 90% for genetically independent isolates (20). Our typing results, comparing two different DNA-based typing methods, also show the largest discordance among genotypically unrelated isolates from patient A. Although RAPD typing may be useful for high-speed typing of *Aspergillus* isolates, the discriminatory power may vary, and therefore RAPD typing should be combined with another DNA-based typing method. Since interlaboratory standardization will be very hard to achieve, we recommend that a collection of well-characterized *Aspergillus* isolates should be available for individual researchers to evaluate new or existing typing techniques, as has been suggested for *S. aureus* (20).

An identical genotype was found among isolates from both patients by RFLP (genotype 4) but not by RAPD. This is difficult to understand, since there was no connection between these patients cross-colonization could not have taken place. Exposition to a common hospital environmental source could be the reason, since on certain occasions a prominent genotype of *A. fumigatus* could be found in the same hospital environment (9a).

In conclusion, this study suggests that different patterns of colonization with *A. fumigatus* exist in CF patients. Since only one isolate from each sputum sample was genotyped, it remains unclear if patients with a greatly fluctuating number of genotypes are colonized with different strains at the same time or that genotypically unrelated *A. fumigatus* strains colonize the respiratory tract successively. To gain more insight into the molecular epidemiology of *A. fumigatus* colonization in CF patients and the effect of antifungal therapy, typing of each cultured isolate is necessary and a greater number of patients need to be analyzed.

We thank A. J. M. M. Rijs and P. van den Hurk for excellent technical assistance and J. B. Yntema and N. Cox, University Hospital Nijmegen, for providing clinical data.

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