JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1994, p. 1115–1118
0095-1137/94/$04.00+0

Vol. 32, No. 4

Fluconazole-Resistant Recurrent Oral Candidiasis in Human Immunodeficiency Virus-Positive Patients: Persistence of *Candida albicans* Strains with the Same Genotype

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Received 15 July 1993/Returned for modification 25 September 1993/Accepted 19 January 1994

Thirty human immunodeficiency virus-positive patients carrying *Candida albicans* in their oropharynx were treated with fluconazole and were monitored for 90 to 570 days. Fluconazole-resistant *C. albicans* (MIC, >32 μg/ml) appeared only in seven patients and only after 90 days of treatment corresponding to a total dose of more than 10 g. Resistance was not associated with resistance to other azole derivatives. Susceptible and resistant strains from each patient had the same genotype (as defined by electrophoretic karyotype and restriction fragment length polymorphism). Thus, the resistant strains were selected by the antimycotic treatment from the susceptible strain present in each case.

Oropharyngeal candidiasis is the most frequent opportunistic infection in patients with AIDS (27). Fluconazole is the best azole drug for the treatment of candidiasis, because its MIC is low, its pharmacokinetic properties are good, and it is well tolerated (6). Of human immunodeficiency virus (HIV) patients with symptoms of candidiasis, 80 to 100% recover within 5 to 10 days of the initiation of fluconazole therapy (2, 4, 6, 10). However, 20 to 100% of such patients relapse within a month once treatment is stopped (2, 3, 6, 10) and the fluconazole resistance of *Candida albicans* strains isolated increases (MICs increase from 6.3 to 100 μg/ml) (7, 8, 11, 20, 28). There is no satisfactory explanation for this phenomenon, and the absence of comparative susceptibility data for isolates recovered before and during treatment does not help identify the origin of the fluconazole insensitivity (16). It is not known whether strains isolated during relapses are mutants of previously present, susceptible strains, or are newly introduced resistant strains. Genotypic characterization is one of the best methods for comparing strains of *C. albicans* (13–15, 17, 24). The genotypic comparison of fluconazole-susceptible and fluconazole-resistant *C. albicans* strains has been reported for only one case (a non-HIV patient after the failure of a 12-day treatment), and only one genotyping method was used (electrophoretic karyotyping by pulsed-field gel electrophoresis) (1).

We report the appearance of fluconazole-resistant strains of *C. albicans* in 7 of 21 HIV-infected patients with oropharyngeal candidiasis, treated with fluconazole for several months. We used two genotyping techniques (electrophoretic karyotyping [EK] by pulsed-field electrophoresis and restriction fragment length polymorphism [RFLP]) (i) to determine the origin of the clinical and fungal relapses and (ii) to distinguish between selection of resistant mutants of *C. albicans* and the implantation of independent resistant strains in patients suffering from candidiasis relapse.

Thirty patients in advanced stages of HIV infection were monitored in the Dermatology Unit of the Besançon Centre Hospitalier Universitaire from November 1990 to November 1992. An oral specimen was taken from each patient at the beginning of the study and every 3 months thereafter. Oral specimens were collected from patients suffering from oropharyngeal candidiasis at the beginning of the treatment (day 0 [D_0]), then at D_7 and D_{30}, and then once a month (the date when the last specimen was taken is designated D_x below). Specimens were collected by vigorous swabbing of the buccal cavity. Infected patients were treated with 50 mg of fluconazole per day, and if no improvement was observed within 1 week, the dose was increased to 100 mg/day.

Specimens were cultured on Sabouraud agar plates (Diagnostics-Pasteur, Marnes-la-Vallée, France) for 21 days at 30°C. The numbers of CFU per swab were counted, and isolates were identified by the development of germ tubes in horse serum. Nonfilamenting *Candida* strains were identified by determining biochemical profiles (ID32C-system; bioMérieux, Marcy l'Etoile, France). Oral candidiasis was diagnosed if the CFU count was more than 50 per swab. The CFU count allowing the diagnosis of oral candidiasis remains a matter of debate (2, 6, 19, 25); we chose a threshold of 50 CFU per swab, because in our experience this value in the conditions we use appears to correlate with pathological oropharygeal candidiasis.

The MIC of fluconazole for each isolate was determined by a microplaque dilution technique (CMI fluconazole; Hoechst-Behring, Rueil-Malmaison, France). Soft medium (mineral solution, pH 7.0, containing 30 g of D-glucose, 2 g of asparagine, 10 mg of L-histidine, 20 mg of DL-methionine, 20 mg of DL-tryptophan, six vitamins, and 15 g of Bacto Agar per liter [Behring-Hoechst]) (5) was inoculated with 10^5 cells per ml (counted in a Malassez cell) and dispatched into microcupules containing a series of concentrations of fluconazole to give final concentrations of 0.25 to 64 μg/ml. The MIC was determined by assessing cell growth after a 48-h incubation at 30°C in a humid atmosphere in comparison with control cell growth (in the absence of antifungal agents). The isolate was scored as having acquired increased resistance if there was at least a twofold increase in the initial MIC. An isolate was considered clinically resistant if the MIC was more than 32 μg/ml. This

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TABLE 1. Clinical and biological data for the thirty HIV-positive patients

Patient group and no.[a]	No. of CD4 cells/mm^3	MIC (μg/ml) of fluconazole on[b]:		Duration (days) of:		TD (g)	Day of appearance of:	
		D_0	D_x	Follow-up (D_0–D_x)	Therapy		*C. krusei*	*T. glabrata*
I								
1	235	4	4	210	0	0		
2	211	4	4	300	0	0		
3	526	4	4	240	0	0		
4	126	4	4	180	0	0		
5	232	4	4	180	0	0		
6	221	4	4	90	0	0		
7	342	4	4	240	6	0.3		
8	400	4	4	90	10	0.7		
9	286	4	4	420	7	0.5		
II								
10	504	4	4	420	4	0.1		
11	234	4	4	120	10	0.7		
12	158	4	4	300	10	0.7		
13	72	4	4	300	37	3.4		D_{180}
14	76	4	4	570	45	4.2		
15	399	4	4	120	48	4.5		
16	26	4	4	240	48	4.5		
17	411	4	4	570	51	4.8		
18	115	4	4	300	90	8.7		
19	77	4	8	330	159	15.6	D_{270}	
III								
20	205	<4	16	150	57	5.3	D_{120}	
21	252	4	16	270	68	6.4	D_{240}	
22	150	<8	16	360	110	10		
23	10	4	16	180	140	13		
IV								
24	12	<4	32	150	120	10		
25	172	<4	64	420	380	19		
26	20	4	64	150	150	16	D_{150}	
27	14	<8	>64	480	380	34		
28	38	4	>64	300	300	30	D_{240}	
29	8	4	32	90	90	36		
30	22	<8	64	150	>100	>10		

[a] Group I, patients colonized by *C. albicans*. Groups II, III, and IV, patients infected with *C. albicans* for which the MIC did not change, for which the MIC increased to 16 μg/ml, and for which the MIC increased to 32 μg/ml and more, respectively.
[b] D_0, day when the first specimen was taken; D_x, day when the last specimen was taken.

endpoint is similar to that used by Dupouy-Camey and coworkers (7). All tests for MICs above 16 μg/ml were done in triplicate. The susceptibilities to seven other antifungal agents were determined by using the Mycototal test (Hoechst-Behring) (5).

Yeast chromosomes were separated by contour-clamped homogeneous electric field electrophoresis in 0.9% agarose gels in a CHEF-DRII system (Bio-Rad, Richmond, Calif.) as previously described (15). For RFLP analysis, yeast DNA was isolated from 5 ml of overnight culture in Sabouraud liquid medium. Cells were harvested in 1 M sorbitol and protoplasted in 1 ml of SCEM buffer (1 M sorbitol, 100 mM sodium citrate [pH 5.8], 50 mM EDTA [pH 7.5], 2% 2-mercaptoethanol) containing 500 IU of lyticase (Sigma Chimie, Saint-Quentin-Fallavier, France). The samples were incubated for 30 min at 30°C. The protoplasts were collected by centrifugation and resuspended in 700 μl of lysis buffer (10 mM Tris HCl [pH 7.5], 50 mM EDTA [pH 7.5], 0.5% sodium dodecyl sulfate, 50 ng of proteinase K per ml) and incubated at 65°C for 30 min. Genomic DNA was precipitated by addition of an equal volume of isopropanol and centrifugation; the pellet was resuspended in 300 μl of Tris-EDTA buffer and treated with 30 μg of RNase for 30 min at 37°C. This mixture was subjected to extraction once with phenol, and the DNA was precipitated by the addition of an equal volume of isopropanol. The resulting DNA was resuspended in a small volume of Tris-EDTA and stored at 20°C until digestion with *Hin*fI restriction enzyme (12) as follows. Yeast DNA (10 μg) was incubated with 20 U of *Hin*fI in a volume of 100 μl for 24 h at 37°C; a further 10 U of enzyme was then added, and the sample was incubated for 24 h at 37°C. The restriction fragments were separated by electrophoresis in 0.7% agarose gels at 80 V cm^{-1} for 3 h.

Clinical and biological data from 30 patients with buccal lesions were collected for between 90 and 570 days (mean, 231 days) (Table 1). Oropharyngeal candidiasis was confirmed in 21 patients (numbers 10 to 30). The patients were classified into 4 groups. Nine patients (numbers 1 to 9; group I) harbored *Candida albicans* (MIC of fluconazole, 4 μg/ml) but did not present candidiasis. All other patients had oral candidiasis and were treated with fluconazole. Ten patients (group II) carried isolates which remained susceptible and for which the MIC of fluconazole did not vary. Four patients (group III) carried

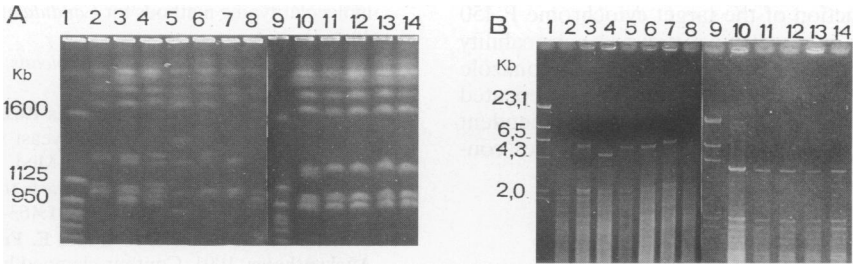

FIG. 1. (A) Electrophoretic karyotypes of *C. albicans*. Chromosomes were separated by pulsed-field gel electrophoresis apparatus (CHEF DRII; Bio-Rad) at 150 V with pulse times of 120 s for 24 h and 180 s for 16 h. Lane 1, *Saccharomyces cerevisiae* DNA; lanes 2 to 8, *C. albicans* DNA from strains isolated from patients number 24 to 30, respectively; lane 9, *S. cerevisiae* DNA; lanes 10 to 14, *C. albicans* DNA from strains isolated from patient number 25 at D_0 (MIC = 4 μg/ml), D_{150} (MIC = 8 μg/ml), D_{240} (MIC = 32 μg/ml), D_{300} (MIC = 64 μg/ml), and D_{360} (MIC = 64 μg/ml), respectively. (B) *Hin*fI DNA restriction fragments from *C. albicans* isolated from group IV patients. DNA (10 μg) was digested with 20 and then 10 U of *Hin*fI. Fragments obtained after a 48-h incubation at 37°C were separated by agarose gel (0.7%) electrophoresis for 3 h at 80 V. Lane 1, *Hin*dIII lambda DNA size markers; lanes 2 to 8: *Hin*fI-digested DNA from *C. albicans* strains isolated from patients 24 to 30, respectively; lane 9, *Hin*dIII lambda DNA size markers; lanes 10 to 14, *Hin*fI-digested DNA from *C. albicans* strains isolated from patient 25 at D_0, D_{150}, D_{240}, D_{300}, and D_{360}, respectively.

strains for which the MIC increased slightly (4 to 16 μg/ml). Seven patients (group IV) carried *Candida albicans* on D_0 with a MIC of fluconazole of 4 μg/ml and on D_x with a MIC of 32 μg/ml or more. The delay between D_0 and D_x was between 90 and 480 days for different patients. The highest MICs for strains from two of the group IV patients were 32 μg/ml, and those for strains from the five other patients in this group were 64 μg/ml or higher. The MICs of the five azole derivatives for the seven fluconazole-resistant strains were each less than 8 μg/ml.

The CD4 lymphocyte counts were higher than 200/mm^3 for eight of the nine group I patients, between 50 and 200 for all the group II patients, and less than 50 for six of the seven group IV patients.

Total doses (TD) of fluconazole were defined as the total fluconazole administered during the study period (D_0 to D_x) (Table 1). The appearance of fluconazole resistance seems to correlate with the TD of fluconazole. As the TD is dependent on the duration of treatment, we compared the MICs for isolates with the administered doses and the frequency and duration of administration for each of the seven group IV patients. Two patients (numbers 28 and 29) carried *C. albicans* with MICs of fluconazole of 4 μg/ml at the start of treatment; the MIC increased to 32 μg/ml after 240 days of 100 mg of fluconazole per day (patient 28) and after 90 days of 400 mg of fluconazole per day (patient 29). Thus, for these cases it seems that fluconazole resistance correlates with the total quantity of fluconazole administered.

A naturally resistant *Candida* sp. (*Candida krusei*) was isolated from 5 patients after 120 to 270 days of treatment (TD, more than 5 g), and *Torulopsis glabrata* was isolated from one patient after 180 days.

The strains isolated from the seven group IV patients (numbers 22 to 29) were genotypically characterized (the average number of strains per patient was six, collected over periods of between 105 and 360 days). Each of the seven patients was colonized by a different strain of *C. albicans* as defined by EK and RFLP (Fig. 1). In contrast, the EK and RFLP of all strains isolated from each patient showed identical banding patterns; the increase of fluconazole MIC was thus not reflected by any variation in EK or RFLP (Fig. 1).

To conclude, clinical and biological investigations confirmed the diagnosis of oropharyngeal candidiasis in 21 of the 30 HIV-infected patients. Fluconazole-resistant *C. albicans* strains were isolated from 7 of these 21 patients.

Fluconazole MIC values depend on several factors, including culture medium composition and pH and inoculum concentration, in addition to the level of resistance of the isolate (9). However, these factors were standardized in the microplaque technique used (5, 7). The increase in the MIC for isolates correlated with the TD of fluconazole: the MIC of fluconazole for the isolates increased to 16 μg/ml for 85% (11 of 13) of the *C. albicans* infections following a TD greater than 5 g; it increased to 64 μg/ml in three patients (numbers 25, 26, and 30) and more than 64 μg/ml in two patients (numbers 27 and 28) following TD of more than 10 g. In contrast, the MICs were below 4 μg/ml for isolates from patients who received a TD of less than 5 g. Fluconazole resistance appeared in the most immunodeficient patients (fewer than 50 CD4 lymphocytes per mm^3); this may have been the case because fungal infections are more frequent and more severe and thus treatment is longer in such patients. It appears, therefore, that a TD of fluconazole of more than 5 g favors the emergence of strains of *Candida* (particularly *C. krusei*) and *Torulopsis* spp. that are less susceptible to this antimycotic agent.

In a study of 29 immunodeficient patients, Pittet et al. (17) demonstrated the persistence of *C. albicans* strains with the same EK pattern over a period of 6 months. We obtained similar results in HIV-positive patients monitored for up to 360 days. Blumberg et al. (1) reported that the MIC of fluconazole for one *C. albicans* strain isolated from one patient increased after a 12-day course of antimycotics and that the EK patterns of pre- and posttreatment isolates were identical, confirming the persistence of the same strain. In our study, we used two independent molecular techniques (EK and RFLP) to confirm that the fluconazole-susceptible and -resistant strains isolated from each of seven patients were the same. It would have been possible to use a third technique (hybridization with the Ca3 probe developed by Soll's group [21]), but the identity between the susceptible and resistant isolates was satisfactorily demonstrated by the two techniques used.

Fluconazole therapy therefore selected resistant mutants for which the MICs increased with the TD. This resistance characteristic is genetically stable after several cultures. However, the fluconazole-resistant *C. albicans* isolates remained susceptible to other azole derivatives. It seems therefore that the resistance mechanism does not involve alterations to 14α-demethylase, a cytochrome P-450 enzyme which is a target of all azole compounds (22). Resistance could be due to alterations of the C5 dehydrogenase involved in ergosterol biosyn-

thesis (26), to overproduction of the target cytochrome P-450 (as in *T. glabrata*, [23]), or to selective reduced permeability (18). The successive increases in the MIC of fluconazole suggest that there were several events, possibly repeated amplification of a gene encoding a target, or independent events associated with different targets. Further studies concerning fluconazole resistance are in progress.

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