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Candida albicans Genotyping in Studies with Patients with AIDS Developing Resistance to Fluconazole

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We characterized *Candida albicans* strains responsible for recurrent oropharyngeal candidosis (OPC) in four patients with AIDS who developed clinical and mycological resistance to fluconazole (FCZ). Karyotype and restriction fragment length polymorphism analyses were performed on the clonal populations to differentiate relapse from reinfection, and the results were assessed with those of serotype and FCZ MICs. Despite the polymorphism in chromosomal bands larger than 2.2 Mbp related to an intraclonal variation, karyotype analysis showed a single strain type attributable to each patient. On the other hand, *Eco*RI and *Hin*fI restriction fragments revealed a polymorphism for one patient between the first sample and the subsequent ones, relevant to the acquisition of a new strain causing the following episodes of OPC. This result coincided with switching of the serotype and with the acquisition of a resistance to FCZ. For the other three patients, the similarity of the DNA electrophoretic patterns and the serotype of the samples suggested that recurrence can be due to the initial strain that generates FCZ resistance. Although useful for epidemiological studies, molecular typing methods seem to be inadequate to detect the acquisition of FCZ resistance.

Oropharyngeal candidosis (OPC) caused by *Candida albicans* is one of the most common opportunistic infections occurring in patients with AIDS (13, 25). Its high prevalence and chronicity has led to improvement in oral treatment allowing for better comfort in these patients. Among the azole drugs, fluconazole (FCZ) showed satisfactory tolerance and efficiency (5, 6). However, increasing resistance with FCZ has recently appeared: it favored the emergence of *Candida krusei* and *Candida glabrata*, which are not frequently isolated from oral lesions, and also the persistence of *C. albicans* (7, 13, 37). These oral recurrent candidiases caused by *C. albicans* can be related to the aggravation of the immune deficit (35) but also to a FCZ resistance phenomenon (14) which was suspected to occur mostly during prolonged primary treatment or prophylaxis of recurrence with low doses (6, 7). Until now, the mechanism of FCZ resistance in *C. albicans* has not been elucidated. Thus, it would be of interest to determine whether a new episode is attributable to the strain responsible for initial infection or to the selection of another strain acquired during treatment.

This purpose necessitated molecular methods consistent with typing of clinical isolates. Merz (18) proposed restriction fragment length polymorphism (RFLP) and karyotype as the most promising methods in clinical studies for strain delineation in comparison with those based on phenotype such as serotype, morphotype, or biotype. However, DNA typing methods are of recent application and lack standardization (18). This could explain the scarcity of epidemiological data, including those in the context of AIDS (2, 23, 27, 35). Nevertheless, RFLP analysis of whole DNA (4, 15, 21, 26, 30, 33) and then electrophoretic karyotype polymorphisms (11, 15, 19, 31) have been described among strains within a single species.

The goals of the present study were to (i) show whether a new episode is caused by relapse or reinfection and (ii) evaluate the simplicity of molecular methods as an epidemiologic tool by using RFLP and karyotype of *C. albicans* oral strains subsequently isolated from four AIDS patients treated with FCZ.

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MATERIALS AND METHODS

Source of *C. albicans*. Four patients with AIDS were selected at the Pasteur Institute Hospital, Paris, France. They were chosen by the following criteria. *C. albicans* was the single species responsible for each OPC episode. An OPC episode was defined as oral swabs were obtained as soon as clinical signs (thrush) and/or symptoms (odynophagia) were detected. Treatment of an OPC episode consisted of oral FCZ at the rate of 100 mg/day for 10 to 15 days, indeed 200 mg/day in a few cases if no improvement was obtained after the first 5 days of treatment. Symptomatological manifestations were generally cleared, but clinical signs were not always eradicated (Fig. 1). The sequence of acute episodes varied from 2 to 18 weeks (Fig. 1) with first relapse or decreased symptomatology intervals that were maintained with FCZ (50 mg/day).

Isolates. Yeast strains from oral swabs were initially isolated and subcultured on Sabouraud dextrose agar at 30°C. All colonies were morphogically homogeneous and identified as *C. albicans* by chlamydospore formation in corn meal agar (Difco, Detroit, Mich.) containing 1% Tween 80 and by their assimilation pattern with the API 20C system (bioMérieux, Marcy l'Etoile, France) (3).

Since a site of infection may be colonized by mixed populations, each culture was analyzed by screening five different mother colonies. Each colony or isolate was suspended in water to 500 CFU/ml, and 100 μl was subcultured to obtain representative clones. Only one clone was arbitrarily selected from each of them for the study, after its identification to the species level was ensured.

Susceptibility to FCZ was determined with the MIC micromethod described by Shawar et al. (29) with the

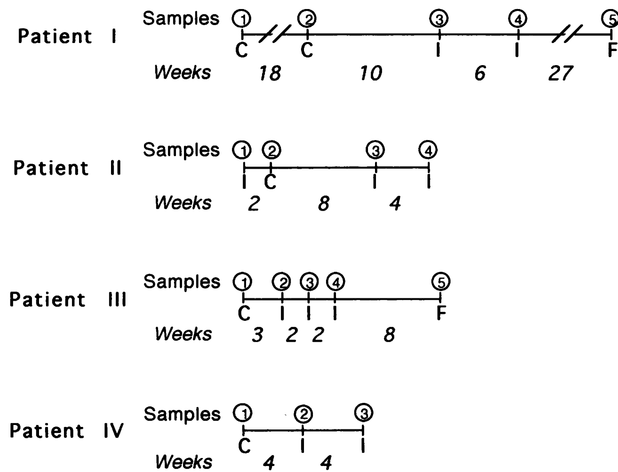

FIG. 1. Time sequence of OPC episodes caused by *C. albicans* treated with FCZ. C, clinical and symptomatological cure; I, clinical and/or symptomatological improvement; F, clinical and/or symptomatological failure; vertical dashes, recovering of an oral swab coinciding with detection of an OPC episode (circled numbers); italic numbers: delay between two OPC episodes.

following modifications. First, 96-well culture plates (Falcon; Becton Dickinson, Paramus, N.J.) were inoculated with chemically defined HR medium (150 μl; a gift from Pfizer Central Research, Sandwich, United Kingdom [22]), a dilution of FCZ solution (150 μl), and yeast inoculum (10 μl). Readings were performed with a Titertek Multiskan spectrophotometer (Flow Laboratories, Inc., McLean, Va.) after 24- and 48-h incubations at 28°C. Yeast cultures were grown in Sabouraud broth overnight at 30°C and suspended in water to obtain a 10^5 ml inoculum. The HR medium was freshly reconstituted and buffered at pH 7.0 with morpholinopropanesulfonic acid (Sigma, St. Louis, Mo.). Fluconazole powder (Pfizer) was dissolved in dimethylformamide to obtain a 1,000-μl/ml solution, and final concentrations tested were 0.09 to 50 μg/ml. Fluconazole MICs for the clones were determined for all samples to evaluate their MIC homogeneity.

All samples were lyophilized before cloning. Yeast clones were stored on Sabouraud dextrose agar at 4°C to prevent multiple subcultures and potentially phenotypic alterations.

Serotyping. The serotype was identified for each clone and each sample by using the Hasenclever and Mitchell 207(A) typing serum prepared at the Centre National de Référence des Mycoses et des Antifongiques, Pasteur Institute, Paris, France (9).

Molecular DNA typing. All experiments were performed on yeast clonal DNA obtained from a single shaken Sabouraud culture grown overnight at 30°C.

Typing by RFLP. Yeast cells were harvested and washed in 0.125 M EDTA (pH 7.5), and the DNA was extracted as described by Holm et al. (10). DNA (5 to 10 μg) was digested overnight at 37°C with restriction endonuclease *Eco*RI and *Hin*fI (2 U/μg of DNA; Boehringer GmbH, Mannheim, Germany). Digestion was halted with a "stop" buffer (33), and electrophoresis of DNA fragments was carried out on 0.8% (wt/vol) agarose gels in 0.04 M Tris-acetate–0.002 M EDTA (pH 8.0) at 40 V for 6 h. *Eco*RI or *Eco*RI-*Hin*dIII digests of bacteriophage λDNA (Boehringer) were included into each gel for molecular size standards. The gels were stained with ethidium bromide solution (0.5 μg/ml) for 30 min and then destained in distilled water for 30 min before being photographed.

TABLE 1. FCZ susceptibility of *C. albicans* samples

Sample	MIC (μg/ml) for patient			
	I	II	III	IV
1	0.36	0.78	3.12	0.78
2	12.5	12.5	12.5	6.25
3	25.0	12.5	12.5	12.5
4	25.0	12.5	25.0	
5	25.0		6.25	

The positions of the major *Eco*RI fragments were precisely determined with the NCSA Gel reader (The National Center for Supercomputer Applications, Champaign, Ill.) in a Macintosh II computer, and then a scheme was drawn with the DNA pattern program of P. Grimont (8).

Karyotyping. The yeast chromosomal DNA molecules were prepared for pulsed-field gel electrophoresis (28) as described previously by Monod et al. (20) and were separated with a CHEF-DR II (Bio-Rad, Richmond, Calif.) apparatus. To obtain the entire karyotype, a 0.6% agarose gel was used, and the ramping pulse regimen of Wickes et al. was employed (36): 120 to 300 s for 30 h, and 420 to 900 s for 66 h, at 70 V. To separate the smaller chromosomes (less than 1.6 Mbp), a 1% agarose gel was used, and the pulse regimen of Monod et al. was employed (20): 120 s for 24 h, and 180 s for 16 h, at 150 V. All runs were at 12°C in 0.45 M Tris–0.45 M boric acid–0.001 M EDTA (pH 7.5). *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Bio-Rad) were the molecular size standards.

Reproducibility. Pattern reproducibility was ensured first by incorporating into each gel *C. albicans* B311 (ATCC 32354) or 3153A (ATCC 28367) as the reference strain since their DNA patterns were previously established (21, 30, 36). Second, for each patient and each sample the patterns of the five clones were compared within a single gel and between gels. Third, karyotypes were performed on three independent series of chromosomal preparations of different clones to confirm the entire method. These controls gave satisfactory results for both DNA patterns performed.

Stability. The stability of DNA electrophoretic patterns was investigated. One clone of each sample belonging to one patient was subcultured every day for 2 months at 30°C by inoculating a 10-ml Sabouraud tube with 20 μl of the last culture, so as to compare parental clones with their descendants after more than 500 generations.

RESULTS

FCZ MIC for clones and samples. The FCZ MICs determined for five clones representing one sample were identical among themselves and with those determined on confluent colonies. The FCZ susceptibility for initial samples varied from the MICs by 0.36 to 3.12 μg/ml (Table 1). The FCZ resistance was defined as an MIC greater than 12.5 μg/ml. Resistance was found from the second or third sample of a given patient. In one case (patient III) the MIC for the last sample decreased to an intermediate level at 6.25 μg/ml.

DNA typing by RFLP. *Eco*RI or *Hin*fI restriction patterns obtained for each patient were identical among the five clones representing one sample and were reproducible. The *Eco*RI digests displayed a complex pattern of bands corre-

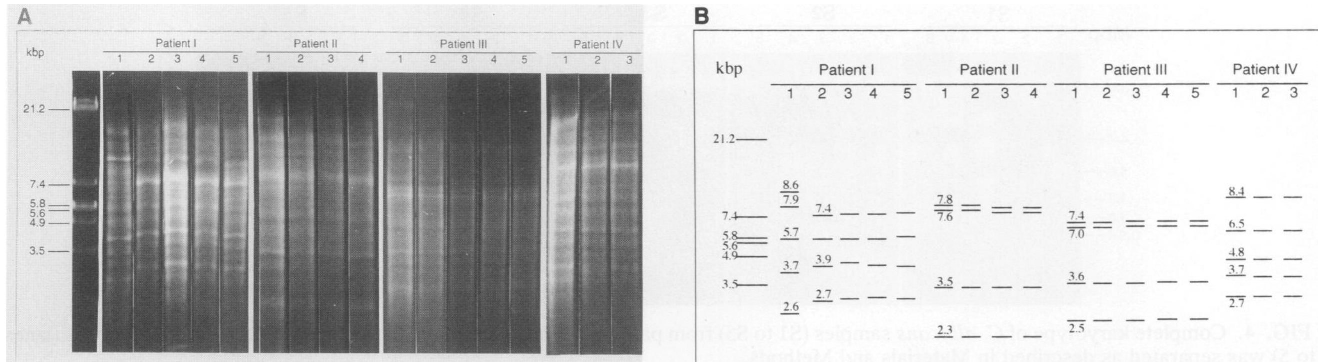

FIG. 2. *Eco*RI restriction fragments. (A) DNA was isolated and extracted as described in Materials and Methods. Digests were run on an 0.8% agarose gel at 70 V and then stained with ethidium bromide. Numbers above the lanes refer to samples subsequently obtained at the beginning of OPC episodes for each patient. The results are illustrated with a single clone for each sample. First lane, *Eco*RI-digested lambda DNA. (B) Scanner representation showing the prominent bands with their respective sizes.

sponding with multiple copies in the nuclear and mitochondrial genomes (Fig. 2). Each sample yielded four to five major fragments: the 3.7- and 2.6-kbp fragments present in all samples; the other fragments, in the size range 6 to 8.5 kbp, allowed the differentiation of a pattern type specific to each patient and revealed an obvious polymorphism among the first sample and the other samples for patient I. *Hin*fI digests consisted of only one or two prominent fragments in the size range 4.4 to 7 kbp (Fig. 3) and led to similar results: patterns of the initial sample assigned only to patient I were obviously different from the subsequent ones, whereas, those obtained from two other patients (patients III and IV) appeared identical.

Identical *Eco*RI and *Hin*fI patterns were recovered when their stability was evaluated after approximately 500 cell cycles.

Karyotyping. The complete chromosomal pattern of *C. albicans* consisted of seven to nine distinct bands ranging from more than 3.0 Mbp to less than 1.0 Mbp. The comparison of the five clonal karyotypes for each sample revealed a polymorphism which concerned chromosomes with sizes of ≥3.0 Mbp. This was encountered for the four patients screened. Figure 4 illustrates the variety of such a polymorphism encountered for a given patient: clonal karyotypes were rarely identical, and some of them could be encountered simultaneously in different samples. Besides, when karyotype stability was investigated, a very similar polymorphism existed. A single clone from each isolate of one patient (patient I) was analyzed after approximately 500 generations. Figure 5 shows that each progeny karyotype (lanes b) was not always identical to the parental one (lanes a). Thus, the chromosomal band at position 2 was recovered in three progeny clones (lanes 1, 4, and 5) but not at the same position as for the parental clones: this band migrated more slowly (lane 4b) or was apparently absent and probably superimposed on another band (lanes 1b and 5b).

Another polymorphism that concerned the smallest chromosomes between 1.3 and 1.0 Mbp was noted. Although less detectable, it allowed the distinction of four kinds of patterns, each corresponding to one patient (data not shown). Also, there was no different between the first sample and the following samples for one patient (patient I). To ensure that there was no difference in chromosomal patterns of each clone, resolution of chromosomes less than 2.0 Mbp in size was specifically performed. No difference was detectable among the clones from each patient (data not shown).

Serotyping. A single serotype was found for all the clones from each sample. For the strains isolated from three patients (patients II, III, and IV) the same serotype was conserved with time. In contrast, the serotype of the samples obtained from patient I switched from A to B during the second OPC episode.

DISCUSSION

We analyzed the karyotype and patterns of restriction fragments for *Candida albicans* strains responsible for OPC in four monitored patients with AIDS who developed clinical resistance to FCZ. This clinical resistance was related to increasing MIC determined by a standardized method tested by Shawar et al. (29). The identity of FCZ MIC determined for the clones was attested by the identity of the clonal RFLP patterns.

Our identification of samples, based on RFLP with *Eco*RI and *Hin*fI, is similar to that of previous investigators (15, 26) who first described this typing method for *Candida* spp. *Eco*RI fingerprints were similar to those found by Whelan et al. (35): three prominent bands (2.5 to 3, 3.7 or 4.2, and 6 to

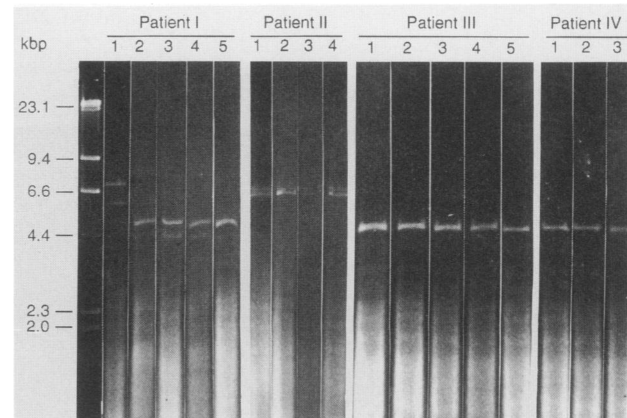

FIG. 3. *Hin*fI restriction fragments. Conditions for DNA extraction and electrophoresis were the same as for Fig. 2. Numbers above the lanes refer to samples subsequently obtained at the beginning of OPC episodes for each patient. The results are illustrated with a single clone for each sample. First lane, *Eco*RI-*Hin*dIII-digested lambda DNA.

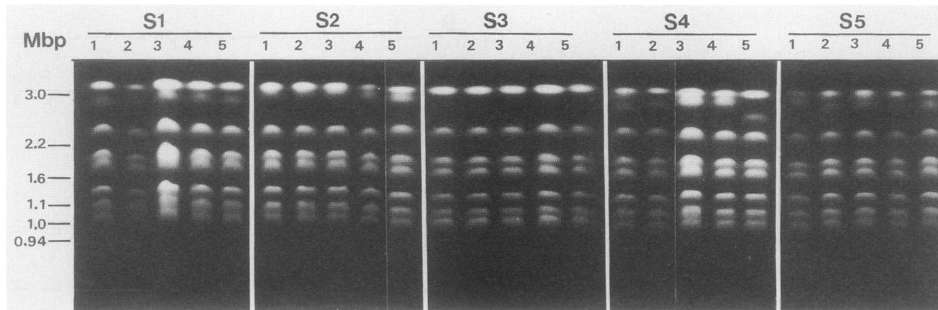

FIG. 4. Complete karyotype of *C. albicans* samples (S1 to S5) from patient I. For each sample, chromosomal DNA from five clones (lanes 1 to 5) was separated as described in Materials and Methods.

7 kbp) appeared to represent ribosomal DNA (rDNA) sequences, and one or two bands (near 8.5 kbp) were presumed to be mitochondrial DNA sequences (38). We did not detect differences in the RFLP in the sequential samples obtained from three patients (patients II, III, and IV) but were able to differentiate the first sample from the four others in one case (patient I). Although differentiation was easier with *Hin*fI, discrimination of samples was not better for each patient, in contrast to findings of other investigators (16, 30, 33): the two prominent bands presumably corresponding to rDNA were less variable in size (4.4 to 7.7 kbp instead of 2 to 10 kbp). This may explain the apparent identity of patterns for two patients (patients III and IV).

The interpretation of the karyotypes was more critical because no strict correspondence exists between the number of chromosomal bands and the number of genetically independent chromosomes of the diploid yeast *C. albicans* (17). We did not know the significance of the polymorphism of the very large chromosomal bands (≥3.0 Mbp) observed within isolates for all patients. The fact that this polymorphism did not allow us to differentiate one patient from another suggested that it was not sufficient to support any strain variation. In this assumption, a similar polymorphism existed for three of the five clones whose DNA was compared before and after 500 generations. This pattern instability has been initially observed by Asakura et al. in sequential vaginal isolates obtained from five women with recurrent vaginitis (1). Iwaguchi et al. (12) recently showed that this polymorphism could occur in clonal progenies with a range of 10% and was probably derived mainly from size heterogeneity in one of the homologs of the MGL1 probed chromosome 2 (1). In our case, this may explain its frequency between and within samples. If the variable chromosomes we observed contain rDNA (36), then this polymorphism reveals an instability of the rDNA among clones. But we did not detect any difference among them with *Eco*RI and *Hin*fI fingerprints, even though these endonucleases possess restriction sites within some rDNA spacers of *C. albicans* (15).

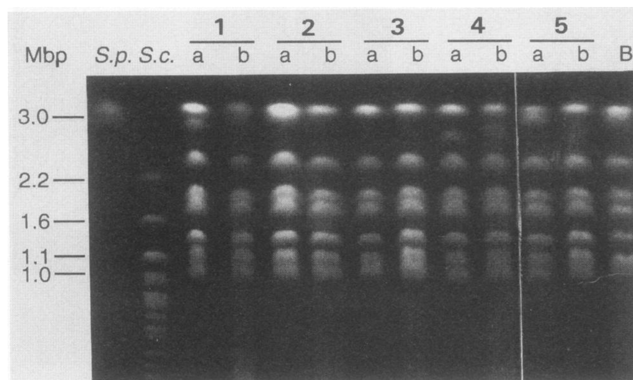

FIG. 5. Study of karyotype stability illustrated with 5 clones (lanes 1 to 5), each derived from the five samples from patient I. Lanes: a, parental clones; b, one of the descendants after approximately 500 cell cycles. *S. p.* and *S. c.*, size markers *S. pombe* and *S. cerevisiae*, respectively. Lane B, *C. albicans* B311 strain.

Also, if distinct strains have caused OPC episodes, karyotypes may have revealed a polymorphism of smaller chromosomes among samples, in particular when an adequate resolution protocol was applied (20). Such a polymorphism was not detected for all four patients or even among clones of one sample, suggesting that each patient was infected with a specific strain and that some variants may have already existed within a single sample.

Otherwise, in comparison with karyotypes, only RFLPs were able to detect the replacement of a strain in one case (patient I). Although our study was limited to very few patients, our findings are in agreement with the findings of Magee et al. (16), who recently concluded that the karyotyping method was probably less discriminatory than RFLP and less practical to use in clinical and epidemiological trials.

The fact that switching of serotype only occurred in one case (patient I) empirically supports the RFLP results and tends to disprove karyotyping performances. Furthermore, if phenotypic modifications have occurred in our study, they may not have corresponded to phenotype switching: although no RFLP has been reported to be associated with switching according to Soll (32), such a morphological change may imply natural chromosomal rearrangements different in frequencies and nature from that observed (24, 34).

In summary, in three cases the original strain was probably maintained while MIC increased rapidly. This situation includes the patient whose MIC decreased from 25 to 6.25 μg/ml (patient III), suggesting that resistance may have been acquired by the strain responsible for the initial episode as far as we know. In contrast, a relationship was found between the strain replacement and the increase of the MIC (12.5 μg/ml) in one case (patient I), suggesting that a new resistant strain could have been selected, in particular by antifungal pressure. This finding is in agreement with the results of Schmid et al. (27) who concluded an early replacement of the original commensal strain to explain the recurrence of *C. albicans* OPC in patients with AIDS, regardless of their pathogenicity. Otherwise, apart from this relation-

ship, the acquisition of FCZ resistance was not supported by electrophoretic karyotype or RFLP. These typing methods were not able to show a potential marker of FCZ resistance. Detection of a hypothetic DNA modification resulting in such FCZ resistance thus belongs to a new research field.

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