JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1995, p. 2732–2737 Vol. 33, No. 10
0095-1137/95/$04.00+0

Fluconazole- and Itraconazole-Resistant *Candida albicans* Strains from AIDS Patients: Multilocus Enzyme Electrophoresis Analysis and Antifungal Susceptibilities

RONAN LE GUENNEC,[1,2] JACQUES REYNES,[2] MICHÈLE MALLIÉ,[1] CLAUDE PUJOL,[1] FRANÇOIS JANBON,[2] AND JEAN-MARIE BASTIDE[1]*

Laboratoire d'Immunologie et Parasitologie, Faculté de Pharmacie, 34060 Montpellier Cedex 1,[1] and Service de Maladies Infectieuses et Tropicales, Hôpital Gui de Chauliac, CHU, 34295 Montpellier Cedex 5,[2] France

Received 29 November 1994/Returned for modification 23 January 1995/Accepted 18 July 1995

Multilocus enzyme electrophoresis and in vitro susceptibility testing with a broth microdilution method were used to analyze *Candida albicans* strain diversity in four AIDS patients with recurrent oropharyngeal candidiasis who successively developed clinical resistance to fluconazole (FCZ) and itraconazole (ITZ). One to ten colonies per sample were randomly chosen from oral washings collected before the initial FCZ treatment and just before every other antifungal treatment; a total of 98 isolates were analyzed. Multilocus enzyme electrophoresis analysis revealed 14 different electrophoretic types (ETs). Statistical analysis of genetic distances showed that *C. albicans* isolates clustered into five subpopulations (I to V). In each subpopulation, isolates are closely related, and genetic distances between subpopulations I to IV are short. In contrast, subpopulation V, which contained isolates typed as ET8 and ET14, is strongly divergent from the others; these isolates may represent atypical *C. albicans* isolates. Only one patient was infected with a single strain during the course of azole therapy; for the three remaining patients, variants of the same strain and different strains were concurrently isolated. Clinical FCZ resistance was clearly correlated with in vitro data for three patients. Moreover, MICs of ITZ increased during FCZ therapy, and MICs of ITZ which were ≥1.56 μg/ml were found when clinical ITZ resistance occurred; isolates from subpopulation V showed the highest MICs of ITZ. Because of the emergence of clinical ITZ resistance after clinical FCZ resistance, the feasibility of long-term azole therapy for mucosal candidiasis in AIDS patients is questioned.

Oropharyngeal candidiasis (OPC) caused by *Candida albicans* is one of the most common opportunistic infections in human immunodeficiency virus type 1-positive patients and is predictive of the development of AIDS (13). Fluconazole (FCZ) has been shown to be effective in the treatment of OPC (7), and the development of recurrent episodes has extended its use during prolonged periods. In the last few years an increasing number of patients with clinical resistance to FCZ were described (2, 8). It was also demonstrated that clinical FCZ resistance may be correlated with in vitro resistance (4, 22, 29).

Recently, biological molecular techniques have been used to study the epidemiology of *C. albicans* infection in AIDS patients (3, 5, 10, 16, 17, 21, 26). Firstly, it was demonstrated that recurrent episodes of OPC could be caused by the same strain during azole therapy (3, 16, 21, 26). However, some investigators have found that AIDS patients could be infected with different strains during successive episodes of OPC (3, 17). Moreover, Pfaller et al. (17) introduced the possibility that these patients could be infected with more than one strain at a given time.

In this study, by using multilocus enzyme electrophoresis (MLEE), which has proven to be a powerful marker system for epidemiological investigations (5, 15, 20), and in vitro susceptibility testing, we have investigated *C. albicans* strain diversity in four AIDS patients with recurrent OPC who successively developed a clinical resistance to FCZ and itraconazole (ITZ).

* Corresponding author. Mailing address: Laboratoire d'Immunologie et Parasitologie, Faculté de Pharmacie, 15, Ave. Charles Flahaut, 34060 Montpellier Cedex 1, France. Phone: 67. 63. 52. 02. Fax: 67. 41. 16. 17.

MATERIALS AND METHODS

Patients. Four human immunodeficiency virus type 1-positive adults attending the AIDS Unit at the University Hospital of Montpellier, France, between September 1990 and August 1993 were investigated. They were monitored for periods ranging from 8 months (patients 2 and 3) to 33 months (patients 1 and 4). The four patients fulfilled the 1987 Centers for Disease Control and Prevention criteria for AIDS. After a clinical cure of a first episode of OPC with FCZ (50 mg daily), all patients received a long-term prophylaxis in continuous use with 50 mg daily of FCZ. The occurrence of clinical relapses required higher dosages of FCZ—up to 400 mg daily. Clinical resistance, defined by the persistence of signs and symptoms of severe OPC despite this dosage for at least 14 days, was observed. A treatment with ITZ solution (100 mg twice daily) was then started. The patients were initially cured, but signs and symptoms of OPC progressively reappeared despite increasing dosages of ITZ—up to 400 mg daily (patients 1, 3, and 4) and 600 mg daily (patient 2).

Isolates. Oral washings obtained with 10 ml of sterile water were collected before the initial FCZ treatment (patients 1 and 4) and just before every other antifungal treatment (Fig. 1); they were plated onto Albicans ID agar plates (bioMérieux, Marcy l'Etoile, France), and the plates were incubated for 48 h at 28°C. Colonies were counted after 48 h, and 1 to 10 colonies of presumed *C. albicans* were randomly chosen (Fig. 1). The ability to produce chlamydospores was tested on Rice Agar Tween (RAT) medium (bioMérieux), and germ tube formation, following growth in a medium for blastesis (Sanofi Diagnostics Pasteur, Marnes la Coquette, France), was studied. Isolates were identified on the basis of chlamydospore production and germ tube formation and by using the ID 32C and the API 20C AUX identification systems (bioMérieux). A total of 98 *C. albicans* isolates were studied.

Antifungal susceptibility testing. Antifungal susceptibility to FCZ and ITZ of *C. albicans* isolates was performed with a broth microdilution method derived from the technique of Annaissie et al. (1). RPMI medium with L-glutamine and lacking sodium bicarbonate (Sigma Chemical Co., St. Louis, Mo.) was used; powder was dissolved in 1 liter of deionized water, and medium was buffered with morpholinepropanesulfonic acid (MOPS) (34.53 g of MOPS per 1,000 ml of water; Sigma Chemical Co.). Medium was titrated with 10 N sodium hydroxide to reach a final pH of 7.3 and then filter sterilized (pore size, 0.2 μm) and stored at 4°C.

Stock solutions of FCZ (Pfizer Central Research, Sandwich, United Kingdom) and ITZ (Janssen Pharmaceutica, Beerse, Belgium) were prepared from reagent-grade powders by the use of deionized water as a solvent for FCZ and

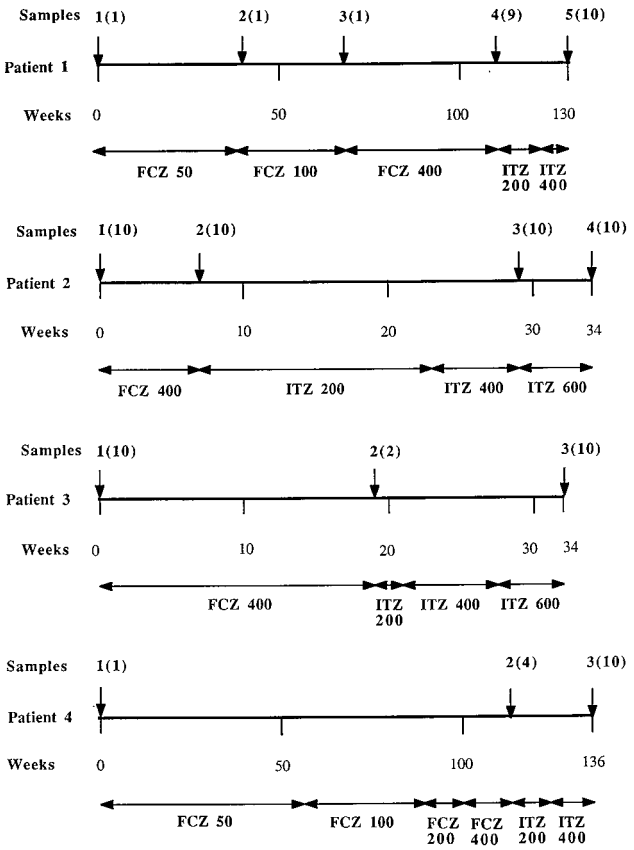

FIG. 1. Sampling procedure and therapeutic course. Above each vertical arrow is the sample number, with the number of colonies randomly chosen in parentheses. FCZ 50, fluconazole at the dosage of 50 mg daily.

100% dimethyl sulfoxide as a solvent for ITZ; initial stock concentrations were 1 mg/ml. The drugs were diluted 10-fold in RPMI medium to provide a working solution for each drug of 100 μg/ml; a second working solution of ITZ (3.12 μg/ml) was prepared.

Broth microdilution susceptibility testing in 96-well microtiter trays was performed as follows. Microtiter plates were inoculated with 150 μl of RPMI medium. Then, 10 twofold serial dilutions were made, and concentrations ranged from 50 to 0.097 μg/ml for FCZ and 50 to 0.097 and 1.56 to 0.003 μg/ml for ITZ. Isolates were subcultured for 48 h at 28°C and picked on Sabouraud agar slants for 18 h before use. Microtiter plates were inoculated with 10 μl of a yeast inoculum of 5×10^5 CFU/ml, and then 20 μl of 2% formaldehyde solution was dispensed into column 1, which served as a blank control for the spectrophotometric assay. Drug-free medium in column 12 served as the growth control. All isolates were tested in duplicate in each plate, and each experiment was repeated one time. Plates were incubated at 28°C for 48 h. Spectrophotometric readings were performed with a UVmax kinetic microplate reader (Molecular Devices) at 490 nm after 5 min of agitation. The MIC end point was defined as the first drug concentration at which turbidity in the well was more than 50% less than that in the growth control well (MIC_{50}). Controls were used to provide reproducibility of the method with two *C. albicans* reference strains, ATCC 90028 and NCPF 3726.

MLEE and data analysis. Isolation of cytosolic extract, starch gel electrophoresis, and enzymatic assays were performed by following the protocols described elsewhere (19). The 11 enzymes assayed were as follows: alcohol dehydrogenase (Adh; EC 1.1.1.1), sorbitol dehydrogenase (Sdh; EC 1.1.1.14), fructokinase (Fk; EC 2.7.1.4), hexokinase (Hk; EC 2.7.1.1), aspartate aminotransferase (Aat; EC 2.6.1.1), leucine aminopeptidase (Lap; EC 3.4.11.1), peptidase 1 (Pep1; EC 3.4.13.18, substrate Val-Leu), peptidase 3 (Pep3; EC 3.4.13.9, substrate Phe-Pro), fumarase (Fum; EC4.2.1.2), mannose-6-phosphate isomerase (Mpi; EC 5.3.1.8), and glucose-6-phosphate isomerase (Gpi; EC 5.3.1.9). Hk enzymatic activity was expressed by two loci (Hk1 and Hk2): thus, data obtained from 11 enzymatic activities corresponded to 12 genetic loci.

Electromorphs of each enzyme, numbered in order of increasing anodal mobility, were equated with alleles at the corresponding structural gene locus. Each isolate was characterized by its combination of alleles at the 12 genetic loci, and distinctive allele combinations were designated electrophoretic types (ETs). Allelic frequencies were used to compute genetic distances between each pair of taxa (9, 12, 28). Furthermore, as the distribution of their estimate fits the normal distribution, we have statistically tested the significance of distances between pairs of taxa. The dendrogram was derived from the distance matrices by using the UPGMA (unweighted pair-group method with arithmetic averages) algorithm, and this information was processed by using a software package called RESTSITE version 1.1 (15a).

RESULTS

Antifungal susceptibilities of *C. albicans* isolates. Antifungal susceptibilities of the reference strains ATCC 90028 and NCPF 3726 were tested in each experiment and were found to be reproducible over time. The FCZ MIC_{50} ranged from 0.78 to 1.56 μg/ml and from 25 to 50 μg/ml for these two strains, respectively; the ITZ MIC_{50} ranged from 0.024 to 0.048 μg/ml and from 0.39 to 0.78 μg/ml, respectively. FCZ resistance was defined as an MIC_{50} of ≥12.5 μg/ml. Antifungal susceptibilities to FCZ and ITZ of *C. albicans* clinical isolates were determined two times; the MIC_{50}s of FCZ and ITZ were the same or differed by only 1 dilution (Tables 1 and 2).

Before the initial FCZ treatment, isolates from patients 1 and 4 were susceptible to FCZ and ITZ. For patients 2 and 3, previously treated with FCZ, the isolates from the first sample showed variable MICs of FCZ and ITZ. For patient 1, when the FCZ dosage increased from 50 to 100 mg daily (sample 2), the MICs of FCZ and ITZ increased to 3.12 and 0.39 μg/ml, respectively; when the dosage was increased from 100 to 400 mg daily (sample 3), the isolate was resistant to FCZ and the MIC of ITZ was 0.78 μg/ml.

When clinical resistance to FCZ occurred, one of nine isolates from patient 1 and three of four isolates from patient 4 were susceptible to FCZ, while the remaining were intermediately susceptible (6.25 μg/ml) or resistant to FCZ. At this time, the MICs of ITZ ranged from 0.006 to 0.78 μg/ml.

During ITZ therapy, isolates from all patients were intermediately susceptible (6.25 μg/ml) or resistant to FCZ. For patient 2, when the dosages of ITZ were increased from 400 to 600 mg daily, the MICs of ITZ for all isolates ranged from 0.195 to 0.39 μg/ml. When clinical resistance to ITZ occurred, 6 of 10 isolates from patient 1, 1 of 10 isolates from patient 2, 4 of 10 isolates from patient 3, and 1 of 10 isolates from patient 4 showed MICs of ITZ that were ≥1.56 μg/ml.

MLEE analysis. Of the 98 isolates studied, all enzyme loci were polymorphic for two (Sdh, Fum, Pep1, Aat, Lap, and Adh), three (Hk1, Hk2, Mpi, and Gpi), or four (Fk and Pep3) alleles. The isozyme pattern was compatible with that expected for a diploid organism. Heterozygotes at the Fk, Hk1, Hk2, Adh, Mpi, and Gpi loci showed two bands, suggesting monomeric proteins; two sorts of heterozygotes were observed at the Fk, Hk1, Hk2 and Mpi loci. Among the homozygotes, two alleles were observed at the Fum, Pep1, Aat, and Lap loci. Three alleles were observed at the Sdh locus, and four were observed at the Pep3 locus.

Of the 12 loci tested, 14 distinctive ETs were identified: allelic makeups of each ET are presented in Table 3. Allelic frequencies were used to compute genetic distances, which were statistically tested (data not shown). These distances were not statistically significant in each subpopulation: ET5, ET6, and ET7 (subpopulation I), ET1, ET2, ET4, and ET9 (subpopulation II), ET10, ET11, ET12, and ET13 (subpopulation III), ET8 and ET14 (subpopulation V). In each subpopulation, isolates differed from one another by only one or two genetic loci. Genetic distances were significant between these four subpopulations and between ET3 (subpopulation IV) and these subpopulations; isolates from two subpopulations differed by three to four genetic loci. The greatest genetic dis-

TABLE 1. Variation in ETs and in MIC_{50}s of FCZ and ITZ for patients 1, 3, and 4[a]

Patient no.	Sample no.	Isolate no.	ET	MIC_{50} (μg/ml)	
				FCZ	ITZ
1	1	1454-1	ET1	0.39–0.195	0.012–0.012
	2	1454-8	ET2	3.12–3.12	0.39–0.39
	3	1454-9	ET2	50–25	0.78–0.78
	4	1454-15A	ET3	0.195–0.195	0.012–0.006
		1454-15B	ET2	>50–50	0.78–0.39
		1454-15C	ET2	50–25	0.78–0.39
		1454-15D	ET2	50–50	0.39–0.195
		1454-15E	ET2	25–25	0.78–0.39
		1454-15F	ET2	>50–>50	0.78–0.39
		1454-15G	ET2	>50–>50	0.78–0.195
		1454-15H	ET2	>50–>50	0.097–0.097
		1454-15I	ET2	50–25	0.78–0.39
	5	1454-22A	ET4	>50–>50	0.78–0.39
		1454-22B	ET4	>50–>50	0.78–0.78
		1454-22C	ET2	>50–50	1.56–1.56
		1454-22D	ET2	>50–>50	1.56–1.56
		1454-22E	ET2	>50–>50	1.56–1.56
		1454-22F	ET2	>50–50	0.78–0.78
		1454-22G	ET2	>50–50	0.78–0.39
		1454-22H	ET2	>50–50	1.56–1.56
		1454-22I	ET2	50–50	1.56–1.56
		1454-22J	ET2	50–25	1.56–1.56
3	1	2029-1A	ET6	3.12–3.12	0.048–0.048
		2029-1B	ET6	3.12–1.56	0.048–0.048
		2029-1C	ET6	0.78–0.39	0.024–0.024
		2029-1D	ET6	3.12–1.56	0.097–0.097
		2029-1E	ET6	0.195–0.195	0.048–0.048
		2029-1F	ET6	1.56–1.56	0.048–0.048
		2029-1G	ET6	6.25–3.12	0.048–0.048
		2029-1H	ET6	1.56–1.56	0.195–0.097
		2029-1I	ET6	6.25–3.12	0.048–0.048
		2029-1J	ET6	3.12–3.12	0.048–0.024
	2	2029-5A	ET7	12.5–6.25	0.048–0.024
		2029-5B	ET7	50–25	0.195–0.097
	3	2029-9A	ET7	12.5–12.5	0.012–0.012
		2029-9B	ET7	6.25–3.12	0.195–0.097
		2029-9C	ET8	50–50	0.78–0.78
		2029-9D	ET7	6.25–3.12	0.195–0.195
		2029-9E	ET7	6.25–3.12	0.048–0.048
		2029-9F	ET7	25–12.5	0.097–0.097
		2029-9G	ET8	50–50	1.56–1.56
		2029-9H	ET8	50–50	1.56–1.56
		2029-9I	ET8	50–25	1.56–1.56
		2029-9J	ET8	50–25	3.12–3.12
4	1	1494-1A	ET9	0.78–0.39	0.012–0.006
	2	1494-12A	ET10	3.12–3.12	0.048–0.048
		1494-12B	ET10	12.5–6.25	0.39–0.39
		1494-12C	ET10	3.12–1.56	0.195–0.097
		1494-12D	ET10	3.12–3.12	0.195–0.195
	3	1494-16A	ET12	50–25	0.78–0.78
		1494-16B	ET13	>50–50	0.39–0.39
		1494-16C	ET11	>50–50	0.78–0.39
		1494-16D	ET11	>50–>50	0.78–0.78
		1494-16E	ET11	>50–>50	0.39–0.39
		1494-16F	ET11	>50–>50	0.39–0.39
		1494-16G	ET11	>50–50	0.39–0.39
		1494-16H	ET14	>50–>50	6.25–3.12
		1494-16I	ET11	>50–50	0.39–0.39

[a] Sample number refers to the sampling procedure (Fig. 1). Isolate number corresponds to the registered number of the isolate in the laboratory.

tance was found between isolates from subpopulation V and the others; these isolates differed from the others by all genetic loci. The genetic relationships between the ETs are presented in a dendrogram (Fig. 2).

The ET distribution for each patient is summarized in Tables 1 and 2. Patient 2 was infected with a single strain (ET5) during the course of azole therapy, while patients 1, 3, and 4 were infected with variants of the same strain and with differ-

TABLE 2. Variation in MIC_{50}s of FCZ and ITZ for patient 2, infected with a unique *C. albicans* strain[a]

Sample no.	Isolate no.	ET	MIC_{50} (μg/ml)	
			FCZ	ITZ
1	2045-1A	ET5	25–25	0.78–0.78
	2045-1A	ET5	25–25	0.78–0.78
	2045-1C	ET5	50–25	0.39–0.39
	2045-1D	ET5	25–25	0.78–0.39
	2045-1E	ET5	50–25	0.78–0.39
	2045-1F	ET5	50–50	0.78–0.39
	2045-1G	ET5	50–25	0.39–0.39
	2045-1H	ET5	50–50	0.78–0.78
	2045-1I	ET5	50–25	0.78–0.39
	2045-1J	ET5	50–50	0.78–0.39
2	2045-2A	ET5	25–25	0.39–0.195
	2045-2A	ET5	25–12.5	0.39–0.39
	2045-2C	ET5	25–12.5	0.39–0.195
	2045-2D	ET5	50–25	0.39–0.195
	2045-2E	ET5	50–50	0.39–0.195
	2045-2F	ET5	12.5–12.5	0.39–0.195
	2045-2G	ET5	50–25	0.39–0.195
	2045-2H	ET5	25–12.5	0.39–0.39
	2045-2I	ET5	12.5–12.5	0.39–0.195
	2045-2J	ET5	12.5–12.5	0.39–0.39
3	2045-11A	ET5	50–25	0.39–0.39
	2045-11B	ET5	50–50	0.195–0.195
	2045-11C	ET5	6.25–6.25	0.39–0.195
	2045-11D	ET5	25–25	0.39–0.39
	2045-11E	ET5	50–25	0.39–0.39
	2045-11F	ET5	12.5–6.25	0.39–0.195
	2045-11G	ET5	12.5–12.5	0.39–0.195
	2045-11H	ET5	12.5–6.25	0.39–0.195
	2045-11I	ET5	6.25–6.25	0.39–0.39
	2045-11J	ET5	6.25–6.25	0.39–0.39
4	2045-13A	ET5	50–50	0.78–0.39
	2045-13B	ET5	50–50	0.78–0.39
	2045-13C	ET5	25–12.5	0.39–0.39
	2045-13D	ET5	>50–>50	1.56–1.56
	2045-13E	ET5	6.25–6.25	0.39–0.39
	2045-13F	ET5	>50–50	0.78–0.78
	2045-13G	ET5	6.25–6.25	0.39–0.39
	2045-13H	ET5	>50–>50	0.78–0.78
	2045-13I	ET5	>50–>50	0.78–0.78
	2045-13J	ET5	50–50	0.39–0.195

[a] Sample number refers to the sampling procedure (Fig. 1), and isolate number corresponds to the registered number of the isolate in the laboratory.

ent strains; these different strains were isolated before FCZ therapy (patient 4, strain typed as ET9), when clinical resistance to FCZ occurred (patient 1, strain typed as ET3), and when clinical resistance to ITZ occurred (patient 3, strain typed as ET8, and patient 4, strain typed as ET14). No correlation between the antifungal susceptibility and the ET for isolates from subpopulations I to IV was found; in contrast, the MICs of ITZ for isolates typed as ET8 and ET14 (subpopulation V) were ≥1.56 μg/ml.

DISCUSSION

We analyzed *C. albicans* strain diversity in four human immunodeficiency virus type 1-positive patients with recurrent OPC who successively developed a clinical resistance to FCZ and ITZ. The MLEE analysis of the 98 isolates revealed 14 ETs, four for patient 1 (ET1 to ET4), one for patient 2 (ET5), three for patient 3 (ET6 to ET8), and six for patient 4 (ET9 to ET14). MLEE provided a powerful typing system because it allowed us to compute genetic distances which were statistically tested; moreover, a dendrogram was derived from the distance matrices to represent the genetic relationships between the isolates. Our analysis of genetic distances allowed us to cluster the 14 ETs into five subpopulations. In each subpopulation, genetic distances were not statistically significant; indeed, ETs differed from one another by only one or two genetic loci. So, isolates from one subpopulation represented variants of the same strain. Common and frequent mechanisms involved in the genetic diversity of *Candida* species could explain this genetic similarity; these include chromosomal rearrangements, chromosomal alterations, and complex and unknown gene regulations. Recently, Rustchenko et al. (23, 24) demonstrated that chromosomal alterations and chromosomal rearrangements could be associated with phenotypic mutants; they also introduced the possibility that these chromosomal rearrangements may affect gene expression (23). Moreover, tandemly repetitive sequences and telomeric and subtelomeric sequences have been described previously (6, 25), and it has been postulated that these sequences may be involved in chromosome organization and rearrangements (25).

In contrast to this genetic similarity, genetic distances between the five subpopulations were statistically significant. These distances between subpopulations I, II, III, and IV are short; isolates from two subpopulations differed by three or four genetic loci and represented two different strains. In contrast, isolates from subpopulation V differed from the others by all genetic loci. Such different *C. albicans* isolates have already been described (18, 27); the authors suggested that they represented atypical *C. albicans* strains. Moreover, Pujol et al. (18) introduced the possibility that these atypical strains may become more represented and selected in AIDS patients, who could represent a favorable environment for their development. In our study, these atypical strains were all isolated during the last stage of human immunodeficiency virus infection and when clinical resistance to ITZ occurred. The state of

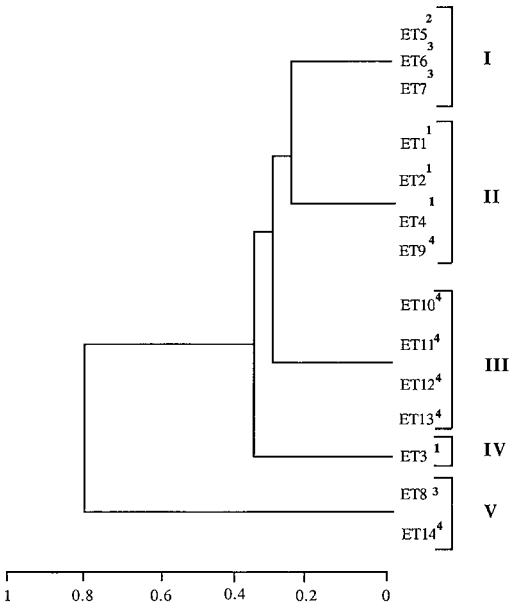

FIG. 2. Dendrogram of *C. albicans* strains derived from the distance matrices by using the UPGMA algorithm. Superscript numbers 1 through 4, patient numbers. I through V, subpopulation numbers.

TABLE 3. Allelic makeup of the ETs

Locus[a]	Allele at indicated enzyme locus[b]													
	ET1	ET2	ET3	ET4	ET5	ET6	ET7	ET8	ET9	ET10	ET11	ET12	ET13	ET14
Sdh	2	2	2	2	2	2	2	0	2	2	2	2	2	1
Adh	1	1	1	1	1	1	1	2	1	1	1	1/2	1/2	2
Fk	1	1	1	1	1/2	1/2	1/2	3/4	2	2	2	2	2	3/4
Hk1	2/3	2/3	1	2/3	1	1/2	1/2	0	2/3	1/2	2	1/2	2	0
Hk2	2	2	2	2	1/2	1/2	1/2	2/3	2	2	2	2	2	2/3
Aat	2	2	2	2	2	2	2	1	2	2	2	2	2	2
Lap	2	2	2	2	2	2	2	1	2	2	2	2	2	2
Pep1	2	2	1	2	2	2	2	1	2	2	2	2	2	1
Pep3	1	1	2	2	2	1	1	4	1	3	3	3	3	4
Fum	2	2	2	2	2	2	2	1	2	2	2	2	2	1
Mpi	2	1/2	3	1/2	1	1	1	0	1/2	3	3	3	3	0
Gpi	1	1	1	1	1	1/2	1	3	1	1	1	1	1	3

[a] Loci encode the enzymes as described in Materials and Methods.
[b] Zero indicates that no enzymatic activity was found at the locus. Heterozygotes having alleles x and y are noted x/y (e.g., 1/2).

immunosuppression, therapeutics, and, in particular, antifungal treatments may account for the emergence of these strains in AIDS patients.

In a previous study, Schmid et al. (26) used DNA fingerprinting with the probe Ca3 and found that isolates from AIDS patients were more closely related than isolates from individuals without AIDS; moreover, they found that sequential isolates from the same patient were highly similar. Our results are in agreement with the results of that study. Indeed, we found that patient 2 was infected with the same strain during azole therapy and that the three remaining patients were infected with variants of the same strain. However, they were also concurrently infected with different strains; these different strains were isolated before FCZ therapy (patient 4) and when clinical resistance to FCZ (patient 1) or to ITZ (patients 3 and 4) occurred. These strains may have been selected from a mixed population or may have been acquired from an exogenous source; although we studied more than one colony per sample, this number is quite limited and did not allow us to choose between these two hypotheses. Recently, Pfaller et al. (17), by using pulsed-field gel electrophoresis, also described AIDS patients with OPC who were infected with more than one strain during the course of therapy.

We also analyzed the antifungal susceptibility of each isolate with a broth microdilution method. In previous studies, it was demonstrated that clinical FCZ resistance may be correlated with in vitro resistance (3, 4, 22, 29). However, Pfaller et al. (17) described AIDS patients with recurrent OPC who were cured with FCZ dosages of up to 400 mg daily despite in vitro FCZ resistance. In our study, we found that clinical resistance to FCZ was correlated with in vitro data for three patients (patients 1, 2, and 3). For the remaining patient, the isolates were susceptible or intermediately susceptible to FCZ when clinical resistance to FCZ occurred; but in contrast to the results reported by Pfaller et al. (17), this patient was not cured with FCZ dosages of up to 400 mg daily.

We also observed an increase in the MICs of ITZ during the course of FCZ therapy: when *C. albicans* isolates were susceptible to FCZ, the MICs of ITZ ranged from 0.006 to 0.097 μg/ml, whereas FCZ-resistant strains showed MICs of ITZ ranging from 0.195 to 0.78 μg/ml. These results are in agreement with the study of Laguna et al. (14), who reported that *C. albicans* isolates resistant to FCZ showed increased MICs of ITZ and ketoconazole (KTZ). It seems that FCZ therapy partially selected less-susceptible isolates to ITZ during FCZ therapy. Several mechanisms have been postulated to explain azole resistance; these include reduced azole membrane permeability (11), mutation in the P-450-dependent 14 α-sterol demethylase (30), P-450 intracellular increased level (31), and reduced sterol C5-6 saturase activity (32). This variety of mechanisms and their combinations may account for the occurrence of partial cross-resistance between FCZ and ITZ during FCZ treatment.

Finally, we described *C. albicans* isolates with MICs of ITZ which were ≥1.56 μg/ml when clinical resistance to ITZ occurred; moreover, all *C. albicans* isolates from subpopulation V showed the highest MICs of ITZ (1.56 to 6.25 μg/ml). According to our experience, it seems that clinical resistance to ITZ is correlated with MICs of ITZ ranging from 0.78 to 1.56 μg/ml. Such high MICs of ITZ were never found during FCZ therapy, and despite the absence of a consensus about the breakpoint of ITZ susceptibility, we assume that *C. albicans* isolates with MICs of ITZ ≥1.56 μg/ml may represent ITZ-resistant isolates and that isolates with MICs of ITZ ranging from 0.39 to 0.78 μg/ml may represent intermediately susceptible isolates.

Our results underscore the great complexity of OPC caused by *C. albicans* in AIDS patients. We found that recurrent episodes of OPC may be caused by the same strain or by variants of the same strain during the course of FCZ and ITZ therapy; moreover, we found that different strains may be isolated during azole therapy. We also demonstrated that (i) clinical resistance to FCZ was correlated with MICs of FCZ of ≥12.5 μg/ml, (ii) MICs of ITZ increased during FCZ therapy, and (iii) isolates with MICs of ITZ of ≥1.56 μg/ml were isolated when clinical resistance to ITZ occurred. Finally, we found that two patients were infected with atypical *C. albicans* isolates, all displaying the highest MICs of ITZ. Despite the first clinical cures of OPC with ITZ after a clinical resistance to FCZ and because of the emergence of clinical resistance to ITZ, the feasibility of long-term azole therapy for mucosal candidiasis in AIDS patients is questioned.

REFERENCES

1. **Anaissie, E., V. Paetznick, and G. P. Bodey.** 1991. Fluconazole susceptibility testing of *Candida albicans*: microtiter method that is independent of inoculum size, temperature, and time of reading. Antimicrob. Agents Chemother. **35:**1641–1646.
2. **Baily, G. G., F. M. Perry, D. W. Denning, and B. K. Mandal.** 1994. Fluconazole-resistant candidosis in an HIV cohort. AIDS **8:**787–792.
3. **Bart-Delabesse, E., P. Boiron, A. Carlotti, and B. Dupont.** 1993. *Candida albicans* genotyping in studies with patients with AIDS developing resistance to fluconazole. J. Clin. Microbiol. **31:**2933–2937.
4. **Cameron, M. L., W. A. Schell, S. Bruch, J. A. Bartlett, H. A. Waskin, and**

J. R. Perfect. 1993. Correlation of in vitro fluconazole resistance of *Candida albicans* isolates in relation to therapy and symptoms of individuals seropositive for human immunodeficiency virus type 1. Antimicrob. Agents Chemother. **37:**2449–2453.

5. **Caugant, D. A., and P. Sandven.** 1993. Epidemiological analysis of *Candida albicans* strains by multilocus enzyme electrophoresis. J. Clin. Microbiol. **31:**215–220.
6. **Chibana, H., S.-I. Iwaguchi, M. Homma, A. Chindamporn, Y. Nakagawa, and K. Tanaka.** 1994. Diversity of tandemly repetitive sequences due to short periodic repetitions in the chromosomes of *Candida albicans*. J. Bacteriol. **176:**3851–3858.
7. **De Wit, S., D. Weerts, H. Goossens, and N. Clumeck.** 1989. Comparison of fluconazole and ketoconazole for oropharyngeal candidiasis in AIDS. Lancet **i:**746–748.
8. **Dupont, B., L. Improvisi, M. Eliaszewicz, G. Pialoux, and the GEMO.** 1992. Resistance of *Candida albicans* to fluconazole (FCZ) in AIDS patients, abstr. 1203, p. 311. *In* Program and abstracts of the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
9. **Gregorius, H.** 1984. An unique genetic distance. Biom. J. **26:**13–18.
10. **Hellstein, J., H. Vawter-Hugart, P. Fotos, J. Schmid, and D. R. Soll.** 1993. Genetic similarity and phenotypic diversity of commensal and pathogenic strains of *Candida albicans* isolated from the oral cavity. J. Clin. Microbiol. **31:**3190–3199.
11. **Hitchcock, C. A., K. J. Barrett-Bee, and N. J. Russel.** 1987. The lipid composition and permeability to azole of an azole- and polyene-resistant mutant of Candida albicans. J. Med. Vet. Mycol. **25:**29–37.
12. **Katz, M., and J. M. Goux.** 1986. The statistical properties of genetic absolute distance. Biom. J. **28:**63–67.
13. **Klein, R. S., C. A. Harris, C. B. Small, B. Moll, M. Lesser, and G. H. Friedland.** 1984. Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome. N. Engl. J. Med. **311:** 354–357.
14. **Laguna, F., E. Valencia, J. V. Martinez-Suarez, J. L. Rodriguez-Tudela, F. Chaves, and F. Dronda.** 1994. Differences between fluconazole-resistant and fluconazole-sensitive oropharyngeal candidiasis in HIV^+ patients, abstr. I213, p. 212. *In* Program and abstracts of the 34th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
15. **Lehmann, P. F., B. J. Kemker, C.-B. Hsiao, and S. Dev.** 1989. Isoenzyme biotypes of *Candida* species. J. Clin. Microbiol. **27:**2514–2521.

15a. **Miller, J. C.** 1990. RESTSITE, version 1.1 package.

16. **Miyasaki, S. H., J. B. Hicks, D. Greenspan, I. Polachek, L. A. MacPhail, T. C. White, N. Agabian, and J. S. Greenspan.** 1992. The identification and tracking of *Candida albicans* isolates from oral lesions in HIV-seropositive individuals. J. Acquired Immune Defic. Syndr. **5:**1039–1046.
17. **Pfaller, M. A., J. Rhine-Chalberg, S. W. Redding, J. Smith, G. Farinacci, A. W. Fothergill, and M. G. Rinaldi.** 1994. Variations in fluconazole susceptibility and electrophoretic karyotype among oral isolates of *Candida albicans* from patients with AIDS and oral candidiasis. J. Clin. Microbiol. **32:** 59–64.
18. **Pujol, C., F. Renaud, M. Mallié, T. De Meeüs, and J.-Marie Bastide.** Unpublished data.
19. **Pujol, C., J. Reynes, F. Renaud, M. Mallié, and J.-Marie Bastide.** 1993. Analyse génétique de souches de *Candida albicans* par électrophorèse des isoenzymes. J. Mycol. Med. **3:**14–19.
20. **Pujol, C., J. Reynes, F. Renaud, M. Raymond, M. Tibayrenc, F. J. Ayala, F. Janbon, M. Mallié, and J.-Marie Bastide.** 1993. The yeast *Candida albicans* has a clonal mode of reproduction in a population of infected human immunodeficiency virus-positive patients. Proc. Natl. Acad. Sci. USA **90:**9456–9459.
21. **Redding, S., J. Smith, G. Farinacci, M. Rinaldi, A. Fothergill, J. Rhine-Chalberg, and M. Pfaller.** 1994. Resistance of *Candida albicans* to fluconazole during treatment of oropharyngeal candidiasis in a patient with AIDS: documentation by in vitro susceptibility testing and DNA subtype analysis. Clin. Infect. Dis. **18:**240–242.
22. **Ruhnke, M., A. Eigler, I. Tennagen, B. Geiseler, E. Engelmann, and M. Trautmann.** 1994. Emergence of fluconazole-resistant strains of *Candida albicans* in patients with recurrent oropharyngeal candidosis and human immunodeficiency virus infection. J. Clin. Microbiol. **32:**2092–2098.
23. **Rustchenko, E. P., D. H. Howard, and F. Sherman.** 1994. Chromosomal alterations of *Candida albicans* are associated with the gain and loss of assimilating functions. J. Bacteriol. **176:**3231–3241.
24. **Rustchenko-Bulgac, E. P., F. Sherman, and J. B. Hicks.** 1990. Chromosomal rearrangements associated with morphological mutants provide a means for genetic variation of *Candida albicans*. J. Bacteriol. **172:**1276–1283.
25. **Sadhu, C., M. J. McEachern, E. P. Rustchenko-Bulgac, J. Schmid, D. R. Soll, and J. B. Hicks.** 1991. Telomeric and dispersed repeat sequences in *Candida* yeasts and their use in strain identification. J. Bacteriol. **173:**842–850.
26. **Schmid, J., F. C. Odds, M. J. Wiselka, K. G. Nicholson, and D. R. Soll.** 1992. Genetic similarity and maintenance of *Candida albicans* strains from a group of AIDS patients, demonstrated by DNA fingerprinting. J. Clin. Microbiol. **30:**935–941.
27. **Sullivan, D., D. Bennett, M. Henman, P. Harwood, S. Flint, F. Mulcahy, D. Stanley, and D. Coleman.** 1993. Oligonucleotide fingerprinting of isolates of *Candida* species other than *C. albicans* and of atypical *Candida* species from human immunodeficiency virus-positive and AIDS patients. J. Clin. Microbiol. **31:**2124–2133.
28. **Thorpe, J. P.** 1979. Enzyme variation and taxonomy: the estimation of sampling error in measurements of interspecific genetic similarity. Biol. J. Linn. Soc. **11:**369–386.
29. **Troillet, N., C. Durussel, J. Bille, M. P. Glauser, and J. P. Chave.** 1992. Fluconazole-resistant oral candidiasis in HIV-infected patients: in vitro-in vivo correlation, abstr. 1202, p. 311. *In* Program and abstracts of the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
30. **Vanden Bossche, H., P. Marichal, J. Gorrens, D. Bellens, H. Moereels, and P. A. J. Janssen.** 1990. Mutation in cytochrome P-450-dependent 14 α-demethylase results in decreased affinity for azole antifungals. Biochem. Soc. Trans. **18:**56–59.
31. **Vanden Bossche, H., P. Marichal, F. C. Odds, L. Le Jeune, and M.-C. Coene.** 1992. Characterization of an azole-resistant *Candida glabrata* isolate. Antimicrob. Agents Chemother. **36:**2602–2610.
32. **Watson, P. F., M. E. Rose, M. E. Ellis, H. England, and S. L. Kelly.** 1989. Defective sterol C5-6 desaturation and azole resistance: a new hypothesis for the mode of action of azole antifungals. Biochem. Biophys. Res. Commun. **164:**1170–1175.