

## In Vitro Susceptibilities and Biotypes of *Candida albicans* Isolates from the Oral Cavities of Patients Infected with Human Immunodeficiency Virus

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*Candida albicans* strains were isolated from the oral cavities of 62 human immunodeficiency virus (HIV)-infected patients at different stages of HIV infection. Only patients with persistent generalized lymphadenopathy-acquired immunodeficiency syndrome (AIDS)-related complex or full-blown AIDS showed typical clinical symptoms for oral candidiasis. In general, the microbiological recovery of *Candida* strains from the oral cavity increased with more advanced stages of HIV infection. The antifungal activity of ketoconazole, itraconazole, nystatin, amphotericin B, and flucytosine against all 62 strains was evaluated by means of a photometer-read broth microdilution method for determination of the 30% inhibitory concentrations of the drugs. The 95% ranges of 30% inhibitory concentrations were as follows:  $\leq 0.063$  to 32  $\mu\text{g/ml}$  for ketoconazole,  $\leq 0.063$  to 8  $\mu\text{g/ml}$  for itraconazole, 0.5 to 4  $\mu\text{g/ml}$  for nystatin,  $\leq 0.063$  to 4  $\mu\text{g/ml}$  for amphotericin B, and  $\leq 0.063$  to 8  $\mu\text{g/ml}$  for flucytosine. Two strains were resistant to flucytosine, one was resistant to ketoconazole, and three were resistant to itraconazole. Isolates from patients with full-blown AIDS showed significantly less susceptibility to itraconazole, amphotericin B, and flucytosine. Strains were biotyped by using the API 20C carbohydrate assimilation system. The major biotype accounted for 63.9% of the isolates. At repeated evaluation, a change in biotype pattern was seen in 27.3%.

*Candida albicans* is the most common causative agent of oral candidiasis in human immunodeficiency virus (HIV)-infected patients (5). During the course of HIV infection, 36 to 88% of all patients suffer from oral candidiasis (4, 15, 21). In one clinical trial, it was shown that the development of oral candidiasis in patients at an early stage of HIV infection might promote the onset of full-blown acquired immunodeficiency syndrome (AIDS) (9).

Despite the high prevalence of oral candidiasis and its relative importance for the further development of HIV infection, no systematic studies of antifungal susceptibility and biotype identification of the *C. albicans* strains involved have been conducted.

The new imidazole derivatives, among them ketoconazole and itraconazole, have been proven highly active against candidiasis in vivo. Conventional in vitro susceptibility testing, however, could never confirm this feature (7, 14) and is therefore of limited value for the evaluation of the antifungal activity of these agents. This is mainly because of partial inhibition of growth over a wide range of drug concentrations (7). As imidazoles such as ketoconazole are among the drugs of choice for the treatment of oral candidiasis in HIV-infected patients, a method has to be employed for antifungal susceptibility testing which avoids the mentioned problem, facilitates large-scale testing, and leads to reliable results both with imidazoles and other antifungal agents. The turbidimetric method first described by Galgiani and Stevens (3) and further developed by Johnson et al. (7) seemed to be very suitable for these purposes. It is a photometer-read broth microdilution method for determination of the 30% inhibitory concentration ( $\text{IC}_{30}$ ), i.e., the lowest drug concentration at which growth is just less than 30% of that in the positive control well.

Although "there is no ideal laboratory procedure or culture medium in current use for susceptibility testing of pathogenic yeasts" (16), this method, in principle, seems to be able to discriminate between those *C. albicans* strains which can be eradicated clinically and the others which cannot (7). This is a major advantage over other tests frequently used, such as the agar dilution test (7; for a more detailed discussion, see reference 11).

The commercially available API 20C carbohydrate assimilation system for yeast identification is widely used in clinical laboratories for biotyping *C. albicans* and serves because of its good reproducibility, sensitivity, and specificity as a reference system for the evaluation of new yeast identification systems (18), although its discriminatory power is limited (22).

In the present report, 88 HIV-infected patients consecutively seen in the outpatient clinic were examined for the oral presence of *C. albicans*. Isolated strains were identified, biotyped with the API 20C system, and evaluated for their susceptibility to five antifungal agents (ketoconazole, itraconazole, nystatin, amphotericin B, and flucytosine) by means of  $\text{IC}_{30}$  determination. The obtained results were correlated with immunological parameters (T4/T8 ratio) and clinical stage of HIV infection.

### MATERIALS AND METHODS

**Patients.** Eighty-eight HIV-infected patients were examined clinically and microbiologically for the presence of oral candidiasis. Clinical staging of these patients was done by the method of a previous study performed on 336 patients in Munich (1). Patients were categorized in three groups: stage 1, seropositive latency; stage 2, persistent generalized lymphadenopathy-AIDS-related complex (PGL-ARC); and stage 3, full-blown AIDS. Seropositivity for HIV was screened by enzyme-linked immunosorbent assay and confirmed by Western blotting (immunoblotting) in each case.

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**Clinical isolates and biotyping.** Sixty-two *C. albicans* isolates were recovered from the oral cavities of the patients. Isolated strains were maintained on Kimmig agar slants at 4°C and subcultured monthly at 37°C for 24 h. Each *C. albicans* strain was identified by using both the Uni-Yeast-Tek system (Flow Laboratories, Meckenheim, Federal Republic of Germany) (18) with an additional germ tube test and the API 20C system (Api Bio Mérieux, Nürtingen, Federal Republic of Germany). The latter was used with the method of Williamson et al. (22).

**Antifungal agents.** The five antifungal agents used for susceptibility testing of *C. albicans* isolates were amphotericin B (Sigma Chemical Co., St. Louis, Mo.), nystatin (Sigma), flucytosine (Sigma), ketoconazole (Janssen, Beerse, Belgium), and itraconazole (Janssen). All the substances were supplied as working standards for laboratory use. Stock solutions were prepared as follows: amphotericin B and nystatin were dissolved in dimethylformamide to a concentration of 3.200 µg of active drug per ml and stored in the dark at 4°C for a maximum of 7 days, flucytosine was dissolved in sterile distilled water to a concentration of 12.800 µg/ml and stored in small portions at -20°C, and ketoconazole and itraconazole were each solubilized in dimethyl sulfoxide substituted with 15 µl of 6 M HCl per ml (12) to a concentration of 12.800 µg/ml and kept at 4°C.

**IC<sub>30</sub> susceptibility testing.** For preparing the inoculum, *Candida* cells were grown to the synchronous stage as described by Soll and Bedell (20) with minor modifications. Blastospores from a 24-h Kimmig agar slant culture grown at 24°C were washed twice and suspended in sterile phosphate-buffered saline, pH 7.4; this suspension was inoculated into modified Sabouraud liquid medium containing 1% (wt/vol) mycological peptone (Oxoid, Wesel, Federal Republic of Germany) and 0.2% (wt/vol) glucose, pH 5.1, resulting in a final cell concentration of 2 × 10<sup>7</sup>/ml, and incubated for 16 h at 24°C in a shaking water bath set at 150 rpm. A portion of this culture was transferred to fresh liquid medium, giving a final cell count of 4 × 10<sup>6</sup>/ml, and incubated under the same conditions for 24 h. Finally, cells were washed three times in phosphate-buffered saline and suspended to a concentration of 10<sup>7</sup> cells per ml.

Serial twofold dilutions of all five antifungal agents were prepared in buffered yeast-nitrogen base (Difco Laboratories, Detroit, Mich.) as described by Shadomy et al. (19). Drug concentrations ranged from 128 to 0.063 µg/ml. Susceptibility testing was performed in sterile round-bottom 96-well microdilution plates (Greiner Labortechnik, Nürtingen, Federal Republic of Germany). Each well was filled with 200 µl of either antifungal solution or yeast-nitrogen base without antifungal solution as a positive growth control. Plates were preincubated at 37°C for 20 min, and 10 µl of the prepared *Candida* suspension in phosphate-buffered saline

TABLE 1. Clinical staging of 84 HIV-infected patients and frequencies of clinical symptoms for oral candidiasis and *C. albicans* recovery from the oral cavity

Stage <sup>a</sup>	No. of patients	No. (%) of patients with:	
		Microbiological proof of <i>C. albicans</i>	Clinical signs for oral candidiasis
1	26	15 (57.7)	0 (0)
2	34	26 (76.5)	12 (35.3)
3	24	21 (87.5)	13 (54.2)
1-3	84	62 (73.8)	25 (29.8)

<sup>a</sup> Stages: 1, seropositive latency; 2, PGL-ARC; 3, AIDS.

TABLE 2. Correlation between manifest oral candidiasis and T4/T8 ratio in 22 HIV-infected patients

T4/T8 ratio	No. of patients (%)
<0.5	15 (68)
0.5-1	6 (27)
>1	1 (5)

was added to each well except the negative growth control wells, which received only antifungal solution. Plates were incubated for 24 h at 37°C and then were read on an MR 600 microplate reader (Dynatech, Denkendorf, Federal Republic of Germany) at a wavelength of 550 nm. IC<sub>30</sub> was the lowest drug concentration at which the recorded optical density was just less than 30% of that in the positive control well. Each test was run in duplicate. As in a former study, a reference strain of *C. albicans* obtained from F. C. Odds, Leicester, United Kingdom, was used (10).

Statistical analysis of IC<sub>30</sub> results was performed by Student's *t* test (17).

RESULTS

**Patient parameters.** *C. albicans* could be isolated from the oral cavities of 62 of 84 patients (73.8%). Of the 62 candida-positive patients, 25 had typical clinical symptoms for oral candidiasis (40.3%), whereas 37 of them were only microbiologically positive, without evidence of *Candida* infection (59.7%). Results of the clinical staging are shown in Table 1. None of the patients with seropositive latency of HIV infection showed clinical signs for oral candidiasis. In patients with PGL-ARC or full-blown AIDS, however, 35.3 and 54.2%, respectively, presented with a typical pattern for oral candidiasis (Table 1). The microbiological recovery of *C. albicans* from the oral cavity increased from 57.7% (seropositive latency) to 87.5% (AIDS) (Table 1).

The correlation between T4/T8 ratios and occurrence of clinical symptoms of oral candidiasis is given in Table 2.

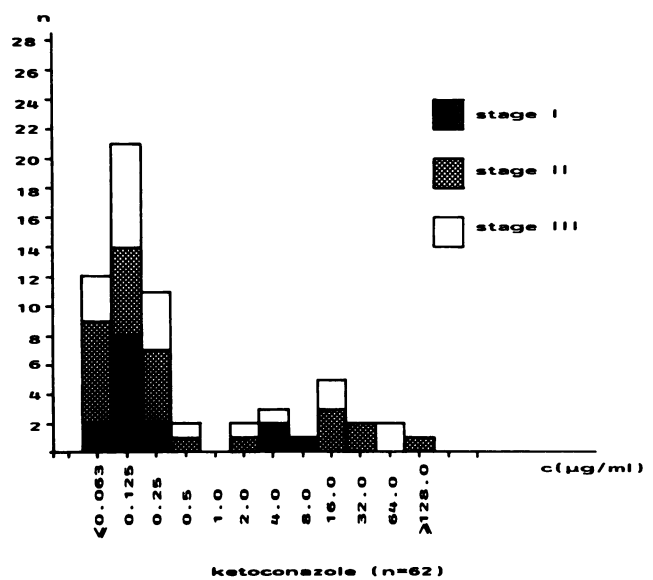


FIG. 1. IC<sub>30</sub>s of ketoconazole for *C. albicans* isolates from HIV-infected patients, with superimposed clinical stages. c, Concentration of drug.

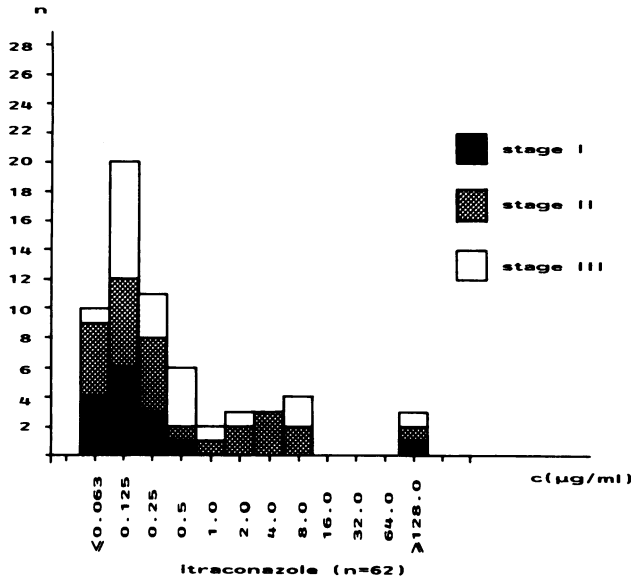


FIG. 2.  $IC_{30}$ s of itraconazole for *C. albicans* isolates from HIV-infected patients, with superimposed clinical stages. c, Concentration of drug.

From these data it is evident that the onset of oral candidiasis in HIV-infected patients correlates well with a reversed T4/T8 ratio. Only one patient with a ratio of >1 had clinical symptoms.

**$IC_{30}$  determination.** The distribution pattern of  $IC_{30}$ s of each antifungal agent against the 62 isolated strains of *C. albicans* is shown in Fig. 1 through 5. Superimposed on each histogram is the clinical stage of HIV infection to which each strain was isolated. The overall mean antifungal susceptibility values for each substance were calculated, and statistical analysis was performed to reveal any significant differences among the three stages of HIV infection (Table 3). Strains isolated from stage 2 and 3 patients were less susceptible to

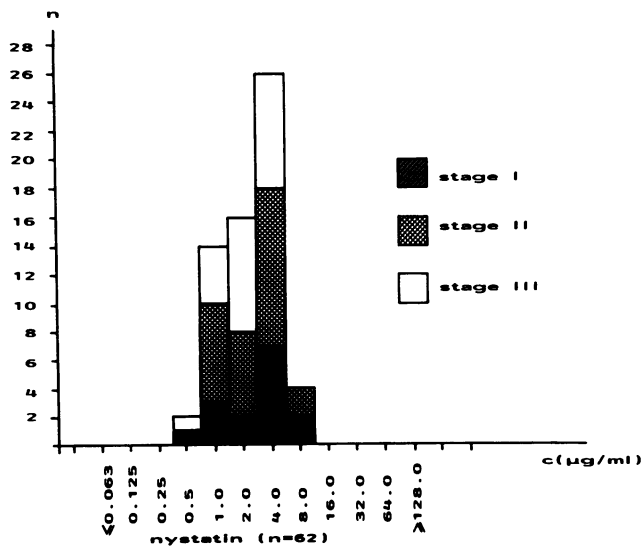


FIG. 3.  $IC_{30}$ s of nystatin for *C. albicans* isolates from HIV-infected patients, with superimposed clinical stages. c, Concentration of drug.

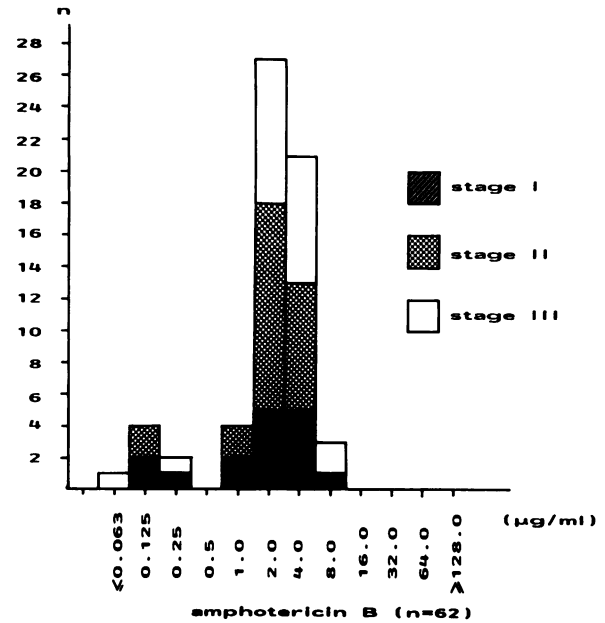


FIG. 4.  $IC_{30}$ s of amphotericin B for *C. albicans* isolates from HIV-infected patients, with superimposed clinical stages.

itraconazole compared with strains from stage 1 patients ( $P=0.025$ ). With amphotericin B and flucytosine, stage 3 isolates showed a significantly lessened susceptibility ( $P=0.025$  and  $P=0.001$ , respectively; Table 3). Resistance to flucytosine was observed with two strains (Fig. 5), to itraconazole with three strains (Fig. 2), and to ketoconazole with one strain (Fig. 1). The 95% ranges for  $IC_{30}$ s were  $\leq 0.063$  to  $32 \mu\text{g/ml}$  for ketoconazole,  $\leq 0.063$  to  $8 \mu\text{g/ml}$  for itraconazole,  $0.5$  to  $4 \mu\text{g/ml}$  for nystatin,  $\leq 0.063$  to  $4 \mu\text{g/ml}$  for amphotericin B, and  $\leq 0.063$  to  $8 \mu\text{g/ml}$  for flucytosine. The cumulative in vitro susceptibilities for all five antifungal agents are given in Fig. 6.

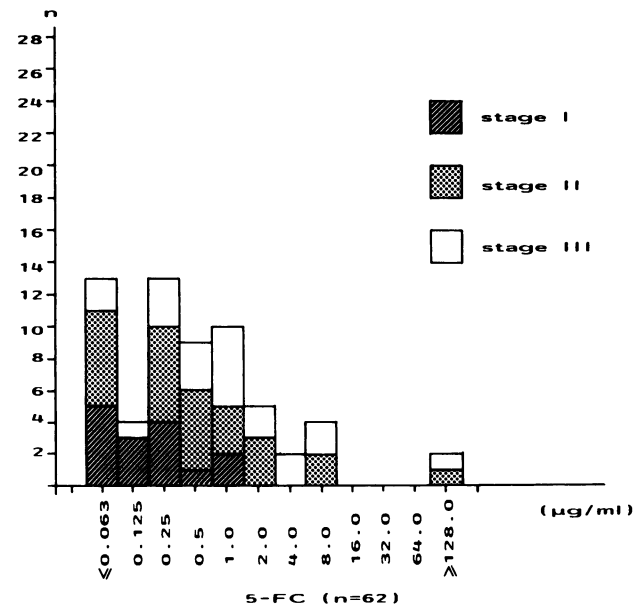


FIG. 5.  $IC_{30}$ s of flucytosine (5-FC) for *C. albicans* isolates from HIV-infected patients, with superimposed clinical stages.

TABLE 3. Geometric means of the inhibitory concentrations of ketoconazole, itraconazole, nystatin, amphotericin B, and flucytosine as determined by IC<sub>30</sub> testing correlated to stage of HIV infection

Stage	Geometric mean inhibitory concn (µg/ml) <sup>a</sup>				
	KCZ	ICZ	NYS	ATB	5-FC
1	0.26	0.21	2.64	1.38	0.17
2	0.49 <sup>b</sup>	0.47 <sup>c</sup>	2.48 <sup>b</sup>	2.00 <sup>b</sup>	0.51 <sup>d</sup>
3	0.53 <sup>b</sup>	0.45 <sup>c</sup>	2.14 <sup>b</sup>	2.28 <sup>c</sup>	0.97 <sup>e</sup>

<sup>a</sup> KCZ, Ketoconazole; ICZ, itraconazole; NYS, nystatin; ATB, amphotericin B; 5-FC, flucytosine.

<sup>b</sup> Not significant.

<sup>c</sup> P = 0.025.

<sup>d</sup> P = 0.05.

<sup>e</sup> P = 0.001.

With the two imidazoles, a higher prevalence of less susceptible strains was observed among patients with clinical symptoms for oral candidiasis. These strains (IC<sub>30</sub>s of ≥8 µg/ml for ketoconazole and ≥2 µg/ml for itraconazole) accounted for 29 and 33%, respectively, of strains recovered in patients with clinical symptoms but for only 11 and 13% in patients without symptoms (data not shown).

**Biotypes.** *C. albicans* strains were biotyped with the API 20C yeast identification system, which measures the carbohydrate assimilation abilities of *C. albicans* (22). The major biotype (B1; positive for xylose, adonitol, xylitol, sorbitol, methyl-D-glucoside, N-acetyl-D-glucosamine, sucrose, and trehalose; negative for glycerol, L-arabinose, and melezitose) accounted for 63.9% of all isolates. Of the minor biotypes (three special biotypes not described by Williamson et al. [22] named B<sub>s</sub>), B<sub>s</sub> was observed in 21.3%. All other biotypes were seen with a frequency of less than 5% (Table 4). A repeated evaluation was possible in 22 patients. A change in biotype pattern was seen in 6 patients (27.3%), whereas 10 patients (45.5%) presented with the same biotype at the follow-up. No significant correlation was found with a single biotype and IC<sub>30</sub>s.

TABLE 4. Biotypes of 61 strains of *C. albicans* as determined by the API 20C system by the method of Williamson et al. (22)

Biotype	No. of strains (%)
B1	39 (63.9)
B2	3 (4.9)
B3	1 (1.6)
B7	2 (3.3)
B8	2 (3.3)
B13	1 (1.6)
B <sub>s</sub>	13 (21.3)

DISCUSSION

The 73.8% recovery of *C. albicans* from the oral cavities of HIV-infected patients determined in the present study is in good concordance with the recovery rates reported by others, which ranged from 36 to 88% (4, 15, 21). Furthermore, the frequency of both cultural proof and clinical signs for oral candidiasis is clearly dependent on the stage of HIV infection, as has been shown recently (21). This observation was confirmed in the present study, revealing a 57.7% microbiological recovery of *C. albicans* from the oral cavity of stage 1 patients, 76.5% from stage 2 patients, and 87.5% from stage 3 patients (Table 1). Additional clinical signs for oral candidiasis were observed only in patients with PGL-ARC and AIDS (35.3 and 54.2%, respectively). The increasing recovery of *Candida* strains from patients with progressive HIV infection should be considered if quantitative laboratory tests are to be taken into account for decisions regarding patient treatment. Kaplan et al. (8) were able to demonstrate that the onset of oral candidiasis in HIV-infected patients is dependent on the absolute T4 cell count. In this trial, only one patient with a T4/T8 ratio of >1 suffered from clinically evident oral candidiasis, whereas all the other patients with clinical signs for oral candidiasis had a ratio of <1.

Somewhat surprising is the result that IC<sub>30</sub>s for flucytosine against *C. albicans* increase with progressive HIV infection.

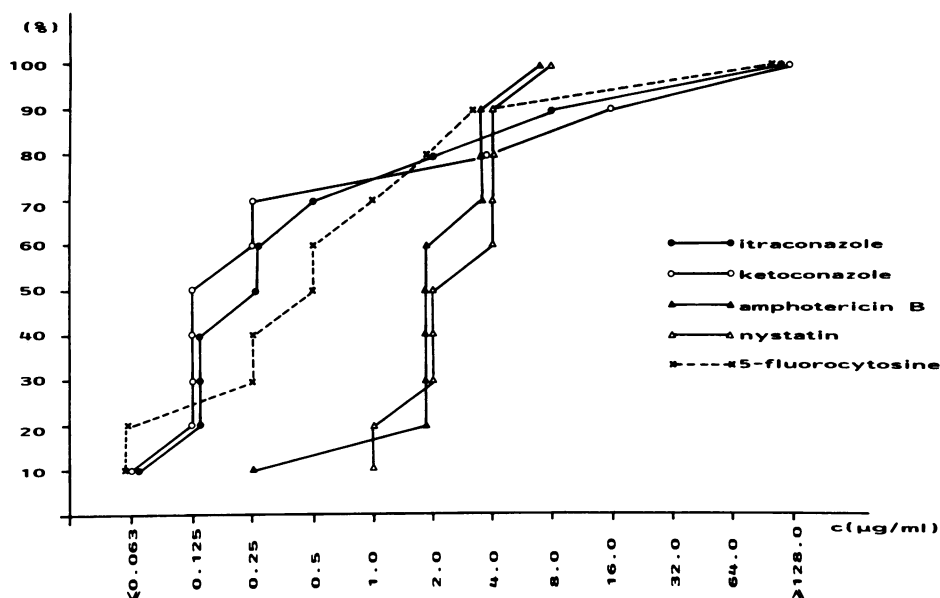


FIG. 6. Cumulative IC<sub>30</sub>s for five antifungal agents against *C. albicans* isolates from HIV-infected patients. c, Concentration of drug.

None of these patients had previous treatment with this antifungal agent, and it appears as if the pattern of less susceptibility to flucytosine is coupled to treatment of candidiasis with other antifungal agents. Many of the patients investigated had already received antifungal treatment during their course of HIV infection. The special ploidy of *Candida* strains which are capable of developing partial or complete resistance to flucytosine (2) might also engender less susceptibility to other antifungal agents. Thus, it needs to be determined whether the gene which encodes flucytosine resistance is linked to genes which are responsible for developing resistance to other antifungal agents.

With both imidazoles tested, a clear tendency to higher  $IC_{30}$ s of *C. albicans* in more advanced stages of HIV infection was observed. While this feature was not statistically significant for ketoconazole, the opposite was true for itraconazole (Table 3). *Candida* strains isolated from stage 2 and 3 patients were significantly less susceptible to the latter. Many of the patients involved in this trial had already received ketoconazole. Although none of them was ever treated with the newer azole itraconazole, susceptibility to this drug was lessened significantly as determined by  $IC_{30}$  testing. One probable reason for lessened susceptibility may be the organism's ability for developing cross resistance to imidazoles. The development of cross resistance of *C. albicans* to different imidazoles during treatment with one single azole derivative has been well described elsewhere (6, 7). This behavior of *C. albicans* has to be kept in mind for the future planning of treatment protocols involving azoles against candidiasis in HIV-infected and other patients.

A more diverse situation was seen with both polyenes, in contrast to the azoles. While no decrease in susceptibility was determined with nystatin, a significant increase in  $IC_{30}$ s of amphotericin B was seen with strains isolated from patients with full-blown AIDS. Although none of the *Candida* strains investigated showed complete resistance to either polyene, the majority of strains could be categorized in an intermediate-susceptibility range (2 to 4  $\mu$ g/ml) for amphotericin B as described by Shadomy et al. (19). This situation is different from that observed with isolates from non-HIV-infected patients, of which the majority of *Candida* strains were highly susceptible to amphotericin B and only a minor percentage showed intermediate susceptibility (19). In a given clinical situation, even these levels might be difficult to obtain on a regular scale by systemic treatment, when the potentially severe side effects of the drug are considered.

The routine application of a reliable and standardized biotyping system for *Candida* isolates from HIV-infected patients is highly desirable for the evaluation and localization of sources of infection, the determination of frequencies of changed biotype patterns, the selection of certain biotypes during therapy, and the differentiation between therapeutic failure and reinfection in cases of recurrent or persistent candidiasis. In this report, the commercially available API 20C system was applied to type *Candida* strains from HIV-infected patients. Unfortunately, as previously stated about strains from HIV-negative patients (22), the distribution of biotypes was very disproportionate, with a single group accounting for 63.9% of all isolates. In this respect, the system is of only limited use for biotyping *C. albicans* isolates from HIV-infected patients. In contrast to the results obtained by Williamson et al. (22), a relatively high percentage of strains typed in the present study (21.3%) revealed distinctive carbohydrate assimilation patterns that were not seen by those authors. To ascertain if this feature is of any importance, additional biotyping systems with a

higher discriminatory power, like the one described by Odds and Abbott (13), must be applied.

In this study, the present susceptibility patterns of clinical *C. albicans* isolates from HIV-infected patients have been described for five major antifungal agents. Long-term antifungal therapy for oral candidiasis in patients with AIDS may result in decreased susceptibility of etiological agents. Data presented here suggest that antifungal-susceptibility studies may assist in the recognition of strains developing resistance during long-term therapy.

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