DEVELOPMENT OF STRAINS OF *CANDIDA ALBICANS* RESISTANT TO CANDIDIN

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ABSTRACT

HEBEKA, ELIAS K. (Rutgers, The State University, New Brunswick, N.J.) AND MORRIS SOLOTOROVSKY. Development of strains of *Candida albicans* resistant to candidin. J. Bacteriol. **84**:237-241. 1962.—Strains of *Candida albicans* resistant to the antifungal agent candidin were developed by growing the organism in gradually increasing amounts of the antibiotic in broth on a shaker. The development of resistance was favored by transfer only after the maximal stationary phase was reached. After 25 transfers, there was a 150-fold increase in resistance. Increased resistance to candidin was accompanied by partial cross resistance to amphotericin B. Strains of *C. albicans* rendered resistant to candidin grew more slowly and were less virulent than the parent cells. The available data favor the hypothesis that the developed resistance is of mutational origin.

The chronic nature of many mycotic infections emphasizes the importance of altered sensitivity of the infectious agent to specific chemotherapeutic agents. Recent interest in amphotericin B and other antifungal antibiotics as therapeutic agents in mycoses has prompted the examination of this problem in several laboratories. *Candida* strains, especially *C. albicans*, have been used for the study of development of resistance to antifungal polyenes. The fungus has often been used as it is one of the common causes of mycotic infections in man, and it is sensitive to polyene antifungal antibiotics. Another useful feature of *C. albicans* is its pathogenicity for the mouse. A number of investigators have encountered difficulties in demonstrating the development of resistance of *C. albicans* to polyene antibiotics (Donovick et al., 1955; Littman, Pisano, and Lanchaster, 1958; Stout and Pagano, 1956). In the present study, strains of *C. albicans* resistant to the heptaene candidin were developed by growing the organism in shaken broth culture and transferring only after the maximal stationary phase had been reached.

MATERIALS AND METHODS

C. albicans strain 204 (obtained from H. Lechevalier, Institute of Microbiology, Rutgers, The State University) was used. It has been maintained by biweekly transfers on Sabouraud's dextrose agar (Difco) slants, incubated at 37 C.

Antibiotics. Candidin was obtained from Carl Schaffner, Institute of Microbiology, Rutgers, The State University. Amphotericin B lot #58126-017, 780 μg/mg, code #22-402 was obtained from the Squibb Institute for Medical Research. Solutions of antibiotics were prepared by dissolving the substance in filter-sterilized dimethyl sulfoxide and diluting to the required volume with sterile distilled water. The solutions were then heated at 80 C for 10 min and cooled. This degree of heat treatment was found adequate for control of contamination. Candidin and amphotericin B were not filtered for sterilization because a portion, at least, of the active components would be lost. Growth of the test organism was not inhibited by the amount of dimethyl sulfoxide used. The stock solutions of the antibiotics were kept in the freezer to a maximum of 10 days (Vining and Taber, 1956). More dilute solutions were prepared and kept at 5 C to a maximum of 5 days (Vining and Taber, 1956).

Cultures were grown in optically uniform 20-mm tubes containing 5 ml of glucose-glycine-yeast extract broth (glucose, 10 g; glycine, 10 g; and yeast extract, 1 g per liter of medium). The tubes were incubated at 37 C on a gyrotary shaker (New Brunswick Scientific Co.) at 200 rev/min until the cultures reached the maximal stationary phase of growth. Where the growth was evaluated turbidimetrically, a Bausch and Lomb Spectronic 20 spectrophotometer was used at 540 mμ.

Virulence test. White female mice (Harpaul) weighing 18 to 20 g were injected intravenously with 0.25-ml amounts of 1:10 dilutions of a suspension of *C. albicans* culture at a turbidity of 85 Klett units with green filter no. 56. This inoculum represented a concentration of 2.3×10^6 cells per ml. The experiments were continued for a period of 30 days. Virulence was estimated from median survival time and from the number of survivors at the end of the test period.

RESULTS

Development of resistance. Subculturing for development of resistant strains was continued for 25 transfers. The first series of tubes was inoculated with 0.1-ml volumes of a cell suspension of the parent cells having an optical density of 0.15. This corresponded to a viable count of 4.3×10^6 organisms/ml. After incubation and adjusting the turbidity to an optical density of 0.15, transfers of 0.1-ml volumes were made from the highest concentration of candidin permitting substantial growth, into similar series of drug dilutions. The antibiotic was increased as permitted by increased resistance of the strain. The highest concentration that allowed at least 50% of the growth, as determined by optical density given by a control treated in the same way but containing no antibiotic, was the point recorded to show the increase in resistance.

For the first 21 transfers, there was a continuous but slow increase in resistance. The rate of increase in resistance was then accelerated. As shown in Fig. 1, after 14 transfers there was a 14-fold increase, and after 25 transfers there was a 150-fold increase in resistance. The parent cells were sensitive to a concentration of 0.5 μg candidin/ml. The cells obtained from the 25th transfer were resistant to 75 μg

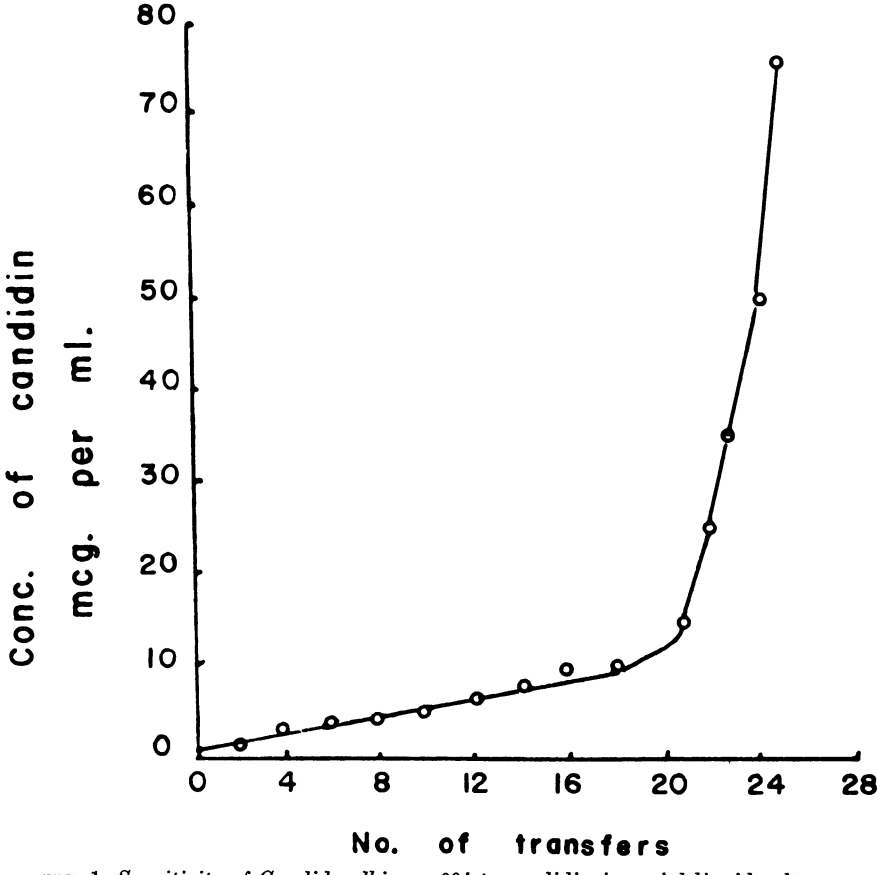

FIG. 1. *Sensitivity of Candida albicans 204 to candidin in serial liquid cultures.*

candidin/ml. A control with the parent cells inoculated into a medium containing 75 μg candidin/ml did not show any growth for 15 days of incubation under the same conditions as the resistant cells.

As resistance increased, the time required for cultures to reach maximal growth was extended. The resistant strains required 72 hr, and the parent cells required 24 hr to give the maximal growth. To show that this increase in resistance was not due to adaptation to the antibiotic, subculturing of the resistant variants was done ten times in the absence of the antibiotic without decrease in the previously acquired resistance. Two other observations confirming this conclusion are the cross resistance and the significant loss of virulence. The results of a fluctuation test (Luria and Delbrück, 1943) also favor the hypothesis that the resistance observed is due to mutation.

Demonstration of cross resistance. Three series of optically uniform 20-mm tubes containing 5 ml of glucose-glycine-yeast extract broth were prepared. One series contained graded concentrations of amphotericin B ranging from 0.05 to 0.7 μg per ml. The second and third series contained graded concentrations of amphotericin B ranging from 0.1 to 3.0 μg per ml. The first series was inoculated with 0.1-ml amounts of inoculum of the parent cell suspension, and the second series was inoculated with 0.1 ml-amounts of inoculum of resistant strains obtained from the 14th transfer (14 times as resistant to candidin as the parent cells). The third series was inoculated with 0.1-ml amounts of inoculum of resistant strains obtained from the 25th transfer (150 times as resistant to candidin as the parent cells). All suspensions were adjusted to permit 75% transmittance. This corresponded to a concentration of 1.5×10^6 cells/ml.

TABLE 1. *Cross resistance between candidin and amphotericin B*

Cells used	Antibiotic (μg/ml)*	
	Candidin	Amphotericin B
Parent	0.5	0.2
Candidin-resistant, 14th transfer	7.0	2.0
Candidin-resistant, 25th transfer	75.0	2.3

* The highest concentration allowing at least 50% of the growth given by the control.

Cells of *C. albicans* 204 resistant to candidin were partially resistant to amphotericin B. As shown in Table 1, there was approximately a tenfold increase in resistance to amphotericin B. By increase of resistance to candidin from 7 to 75 μg/ml, there was no further significant increase in resistance to amphotericin B.

Virulence test. A group of 30 mice was injected with a suspension of culture of the original sensitive strain. Another group of 30 mice was injected with a suspension of culture of the resistant strains obtained from the 14th transfer. A group of ten mice was used as uninfected controls. The more resistant strains were less virulent than the parent cells. At 30 days after

TABLE 2. *Relation between resistance to candidin and virulence of Candida albicans*

Organism injected	Culture dilution	Median survival time	Survivors/total at 30 days	Survivors
Strains with 14-fold increase in resistance				%
C. albicans, parent strain	Undiluted	13	4/30	13.3
C. albicans, resistant strain from 14th transfer	Undiluted	>30	20/30	66.7
Uninfected controls	Undiluted	—	10/30	100.0
Strains with 150-fold increase in resistance				
C. albicans, parent strain	Undiluted	15	2/10	20
	1:5	20	5/10	50
	1:10	—	8/10	80
C. albicans, resistant strain from the 25th transfer	Undiluted	—	8/10	80
	1:5	—	10/10	100
	1:10	—	10/10	100
Uninfected controls	—	—	10/10	100

infection, 4 of 30 mice challenged with the parent strain survived as compared with 20 survivors of 30 mice challenged with the resistant strains (Table 2).

When this experiment was repeated 1 year later, using resistant strains obtained from the 25th transfer, it was noticed that the virulence of the original stock culture of *C. albicans* 204 decreased. Hence, three levels of infection were used, namely, undiluted, 1:5, and 1:10 dilutions. The undiluted cell suspension had an optical density of 0.13, which corresponded to a viable count of 3.9×10^6 organisms per ml. The conditions of the experiment were the same as before with the exception of decreasing the number of mice per group from 30 to 10 mice. The percentage of survivors of mice challenged with the parent strain, at 30 days after infection, was 20%, compared with 80% in the case of mice challenged with the resistant strain (Table 2). It is clear that, despite the difference in resistance to candidin between cells obtained from the 14th transfer and those obtained from the 25th transfer, the difference in virulence of the two strains was not significant.

DISCUSSION

Previous investigators have reported that high resistance to polyene antibiotics was not attained by *C. albicans* (Donovick et al., 1955; Littman et al., 1958; Stout and Pagano, 1956). A consideration of the methods of these investigators led to the following modifications in procedures that have been used in the present report.

When grown in liquid medium, *C. albicans* cells tend to clump at the bottom of the tube. It would appear possible that antibiotic might not penetrate readily into the center of the clumps; under such circumstances, the selective advantage for resistant mutants would have been reduced. Sorenson, McNall, and Sternberg (1959) used shaken cultures, and they observed the development of a high degree of resistance of *C. albicans* to amphotericin B. Two of the strains they used were partially inhibited by 0.1 μg/ml, and after 58 transfers they were not completely inhibited by 1,000 μg/ml of amphotericin B.

In addition to shaking to disperse clumps, the incubation of test cultures was continued until the maximal stationary growth phase was reached. Cells isolated from the logarithmic phase may show a greater variability with regard to growth in the presence of the antibiotic than those isolated during the maximal stationary phase (Drobnica, 1960; Hebeka, 1960).

Regarding the binding of the antibiotic by the sensitive microorganisms, Lampen et al. (1959) found that microorganisms whose growth was sensitive to nystatin absorbed significant amounts of the antibiotic. Also, Pledger (1957) showed that there was an uptake of candidin by *C. albicans* and *Saccharomyces cerevisiae*, both sensitive to the antibiotic, whereas bacteria not sensitive to the antibiotic did not significantly alter the concentration of the antibiotic. Inocula for serial transfers in development of resistance were taken from tubes of comparable density and standardized carefully in an attempt to obtain similar rates of growth and, consequently, similar degrees of binding of the antibiotics.

With these three modifications, strains of *C. albicans* were developed that were 150-fold resistant to candidin after 25 transfers. The rate of development was comparable to the pattern observed for multistep development of resistance by various antibiotics, including amphotericin B as described by Sorenson et al. (1959).

Cross resistance among polyene antibiotics is recognized. Sorenson et al. (1959) and Littman et al. (1958) have recorded the interrelationship between amphotericin B and nystatin. The former investigators used strains of *C. albicans* resistant to amphotericin B and observed five- to eightfold increases in resistance to nystatin, but not pimaricin. Littman et al. (1958) found that some, but not all, of the species of *Candida* resistant to nystatin were also resistant to amphotericin B and vice versa. The order of the cross resistance varied from 1.5- to 50-fold as compared with the original cultures. Under our conditions, the cross resistance between candidin and amphotericin B was partial.

Significant alterations in virulence occurring with development of resistance has been observed with bacteria. Catalase-negative, isoniazid-resistant mutants of *Mycobacterium tuberculosis* may be less pathogenic than the original isoniazid-susceptible strains (Middlebrook, 1956). In our study, strains of *C. albicans* resistant to candidin were less virulent for mice than the parent sensitive strain. This agrees with the observations of Lones and Peacock (1959), who

found the same relation with strains of *C. albicans* resistant to amphotericin B. They used two strains that were 13- and 29-fold resistant, and they found a significant decrease in virulence for mice. However, repeated subculturing in vitro might favor the selection of less virulent strains, independent of the presence of the antibiotic.

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