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Development of Resistance to Polyene Antibiotics in *Candida albicans*[1]

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ABSTRACT

HEBEKA, ELIAS K. (Rutgers, The State University, New Brunswick, N.J.), AND MORRIS SOLOTOROVSKY. Development of resistance to polyene antibiotics in *Candida albicans*. *J. Bacteriol.* **89**:1533–1539. 1965.—Strains of *Candida albicans* resistant to the polyenes candidin and amphotericin B, but not to nystatin, were developed by subculturing the organism in gradually increasing concentrations of the antibiotic in broth on a shaker, or by repeated transfer on gradient plates. Demonstration of resistance on solid media was best observed when a purified agar, Ionagar no. 2 (Oxoid), was used in preparing the medium. Strains that were 150-fold resistant to candidin, and 4-, 16-, 45-, and 60-fold resistant to amphotericin B were developed. The degree of resistance depended on the strain, the type of medium, and, most importantly, on the antibiotic used. The polyenes candidin, amphotericin B, nystatin, and fungimycin and the nonpolyenes griseofulvin and eulicin were used to extend the scope of study of cross-resistance. Cells rendered resistant to candidin were also resistant to amphotericin B, but not to nystatin, fungimycin, or griseofulvin. Cells rendered resistant to amphotericin B showed cross-resistance to candidin, but not to nystatin, fungimycin, or griseofulvin. Candidin- or amphotericin B-resistant strains were more sensitive to eulicin than their parent strains. Increased resistance to candidin or amphotericin B was accompanied by a decrease in virulence for mice, the rate of growth, the ability to reduce bismuth sulfite, and by an increased tendency for filamentation. No change in the ability to form chlamydospores was noticed.

In a previous paper (Hebeka and Solotorovsky, 1962), we reported on a successful attempt to develop strains of *Candida albicans* resistant to the heptaene candidin. The organism was grown on a shaker in gradually increasing amounts of the antibiotic. The development of resistance was favored by transfer only after the maximal stationary phase was reached. The present study has extended the knowledge of development of resistance to the polyenes amphotericin B, nystatin, and candidin by use of different strains of *C. albicans* in liquid and on solid medium. Changes in virulence, the rates of growth, and other characteristics correlated with the development of resistance were also studied.

MATERIALS AND METHODS

C. albicans 204, 211, and 452 were used. All strains were obtained from the culture collection of the Department of Bacteriology, Rutgers, The State University. All strains were maintained by transfers on Sabouraud Glucose Agar (Difco) slants, incubated at 37 C for 24 hr and held at 4 C for 2 months.

Media. Sabouraud Liquid Medium (Difco) was used for studies in broth culture. When a solid medium was needed, Sabouraud Glucose Agar was used. Two different kinds of agar were used: Difco agar and Ionagar no. 2 (Oxoid).

Antibiotics. Candidin, nystatin, amphotericin B, fungimycin, griseofulvin, and eulicin sulfate were tested during the course of the study. Candidin, batch 3, was obtained from Carl Schaffner of the Institute of Microbiology, Rutgers, The State University. Nystatin and amphotericin B were provided by the Squibb Institute for Medical Research, New Brunswick, N.J. Nystatin, 780 μg/mg, was used. Fungimycin (WXg2412; formerly known as perimycin) was provided by Warner-Lambert Research Institute, Morris Plains, N.J. Griseofulvin (UGR 873) was obtained from Schering Research Division, Bloomfield, N.J. Eulicin sulfate in the form of sterile aqueous solution that contained 5 mg/ml was provided by Merck Sharp and Dohme Research Laboratories, Rahway, N.J. All the antibiotics, except eulicin, were brought into solution by dissolving them in dimethylsulfoxide that was sterilized by filtration. Further

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dilutions were made with sterile distilled water. Eulicin sulfate was diluted with sterile distilled water. The antibiotic solutions were prepared in four different concentrations: 0.05, 0.50, 1.0, and 5.0 mg/ml. The solutions were heated at 80 C for 10 min and cooled. The stock solutions (the highest concentrations) of the polyenes were kept in a freezer and were not held longer than 20 days. The more dilute solutions were kept at 4 C for a maximum of 10 days (Vining, 1960). The degree of heat treatment used was found adequate for control of contamination.

For development of resistance in liquid medium, the method previously described by Hebeka and Solotorovsky (1962) was used. Glucose-glycine-yeast extract was replaced with Sabouraud broth. The highest concentration of the antibiotic that allowed visible growth after 72 hr of incubation was the point recorded to show the increase in resistance.

For development of resistance on solid medium, the gradient plate technique (Szybalski, 1952) was used. Each layer consisted of 20 ml of Sabouraud Glucose Agar. The antibiotic was added to the upper layer only. Two plates were used for each concentration, and two streaks were prepared on each plate. The inoculum was obtained from *C. albicans* cells that were grown in Sabouraud Liquid Medium on a shaker until they reached the stationary phase of growth. All inocula for streaking had an optical density (OD) of 0.35 at 540 mμ (approximately 8×10^6 viable cells per ml). The plates, after streaking, were incubated at room temperature for 48 hr. The concentration of antibiotic in the upper layer of agar was increased as permitted by increased tolerance of the strain. The increase in resistance was determined by measuring the length of a streak of growth and multiplying this number by concentration of antibiotic in the plate. All turbidimetric measurements were made with a spectrophotometer (Coleman Jr. 6A) at 540 mμ, unless otherwise specified.

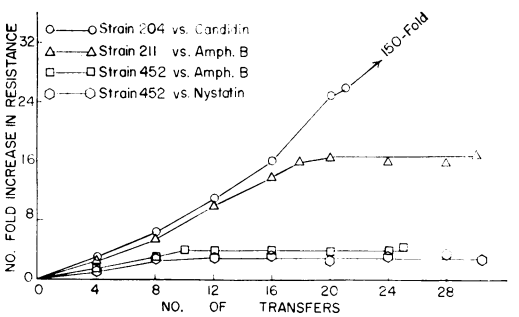

FIG. 1. *Development of resistance of strains of Candida albicans to polyene antibiotics in liquid cultures. (i) Strain 204 and candidin: initial resistance, 0.5 μg/ml; final resistance, 75 μg/ml. Resistant strain was designated as strain 215. (ii) Strain 211 and amphotericin B: initial resistance, 3.0 μg/ml; final resistance, 48 μg/ml. Resistant strain was designated as strain RM 211. (iii) Strain 452 and amphotericin B: initial resistance, 2.5 μg/ml; final resistance, 10 μg/ml. Resistant strain was designated as strain 216. (iv) Strain 452 and nystatin: initial resistance, 4 μg/ml; final resistance, 12 μg/ml. Resistant strain was designated as strain RM 452.*

Demonstration of cross-resistance. Cultures grown in Sabouraud glucose broth and adjusted to an OD of 1.0 (approximately 2×10^7 viable cells per ml) were streaked on Sabouraud Glucose Agar containing appropriate concentrations of antibiotic. Ionagar no. 2 was used, and all plates were incubated at room temperature for 72 hr.

Virulence test. White, Swiss, female (Harpaul) mice weighing 18 to 20 g each were injected intravenously with 0.25 ml of suspensions of *C. albicans* cultures adjusted to densities to permit determinations of virulence. Strains 204 and 452 were adjusted to an OD of 0.13 (a viable count of 2.3×10^6 cells per ml) and strain 211 to an OD of 0.4 (a viable count of 9.0×10^6 cells per ml). The resistant strains derived from the parent strains were adjusted to the same OD. Wherever possible, a *C. albicans* strain that was transferred the same number of times as the resistant one, but in the absence of antibiotic, was also used to infect mice. This was a control for the effect of transferring *C. albicans* cells in vitro on virulence of the organism. Virulence was estimated from median survival time and from the number of survivors at the end of the test period.

Alterations in reactions characteristic of C. albicans strains. To determine changes in morphology, the organism was grown in Sabouraud Liquid Medium in stationary and shake cultures. It was also grown on Sabouraud Glucose Agar. The growth of the parent and resistant strains was compared macroscopically and microscopically.

To determine changes in chlamydospore formation, Chlamydospore Agar (Difco), as described by Nickerson and Mankowski (1953), was used. A slide culture technique described by MacDonald and Wegner (1962) was employed. The slide cultures were inoculated heavily and incubated at room temperature for 4 days.

C. albicans strains were grown on Bi GGY agar (Difco; described by Nickerson, 1953). Freshly prepared plates were streaked with suspensions of the parent and resistant strains adjusted to an OD of 0.35 (8×10^6 viable cells per ml). The cultures were incubated at room temperature for 48 hr or longer (as explained in a later section). Strains that were transferred the same number of times as the resistant ones, but in absence of the antibiotic, were used as controls for the effect of transferring *C. albicans* cells in vitro on their ability to reduce inorganic sulfite.

RESULTS

Development of resistance by subculturing in liquid medium. The results of the studies of the development of resistance to candidin, amphotericin B, and nystatin are presented in Fig 1. Strain 204 developed progressive resistance to candidin.

Trials were discontinued when a 150-fold increase was attained. Two strains were studied for development of resistance to amphotericin B. The resistance of strain 211 rose 16-fold and persisted at this level. Strain 452 showed only a threefold rise in resistance to nystatin.

All the resistant strains were passed 10 times in Sabouraud broth in absence of antibiotic. The level of resistance was again checked, and no significant loss of resistance was observed.

Subculturing on solid medium. The first attempts to develop resistant strains by the gradient plate technique were unsuccessful. At the beginning, Sabouraud medium was prepared with Difco. From the pattern of growth of the organism, it appeared that the antibiotic diffused poorly. In an attempt to obtain a better diffusion of the polyene, a purified agar, Ionagar no. 2 (Oxoid), was used in preparing Sabouraud agar medium. The improved diffusion is shown in Fig. 2A. On amphotericin B, both strains gave a "tapering" pattern of growth; on nystatin, the pattern was "abrupt" (Fig. 2B). Both strains were transferred 20 times on plates containing nystatin or amphotericin B, and their behavior was almost identical (Fig. 3). After 20 transfers on amphotericin B, strain 452 showed an approximately 60-fold increase, and strain 211 showed a 45-fold increase. With nystatin, as in the case of

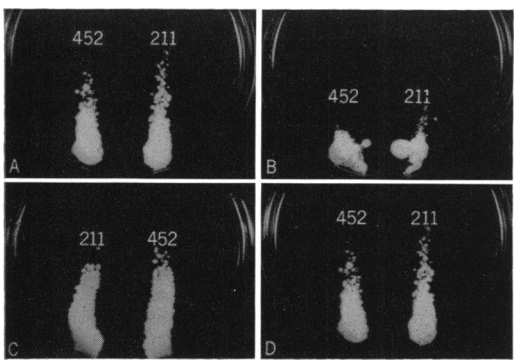

FIG. 2. *(A and B) Growth of Candida albicans 211 and 452 on gradient plates containing 60 μg of amphotericin B per ml. (A) Sabouraud medium was prepared with Ionagar no. 2 (Oxoid), and (B) Sabouraud medium was prepared with Difco agar. (C and D) Appearance of growth of C. albicans 211 and 452 on the following: (C) nystatin, 20 μg/ml; (D) amphotericin B, 60 μg/ml.*

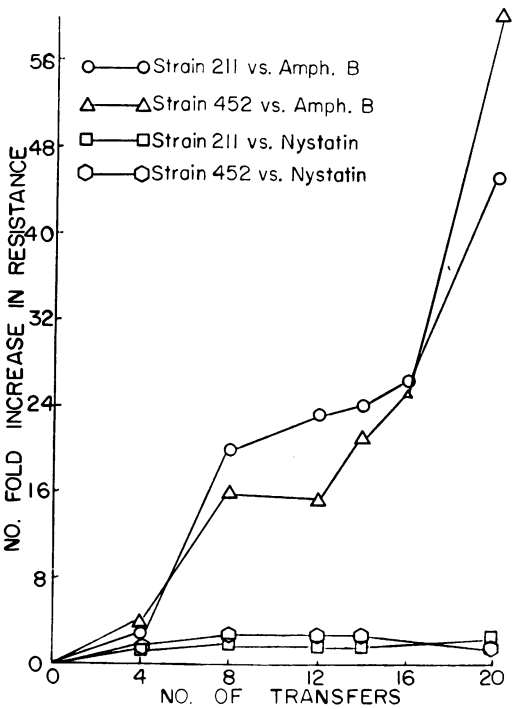

FIG. 3. *Development of resistance of Candida albicans to polyene antibiotics on gradient plates.*

TABLE 1. *Demonstration of cross-resistance on solid medium*

Candida albicans strain	Resistant to	MIC*					
		Amphotericin B	Nystatin	Candidin	Fungimycin	Griseofulvin	Eulicin sulfate
204	—	5†	10	5	35	500	90
215	Candidin	75	15	75	35	500	40
452	—	10	10	10	50	450	100
216	Amphotericin B	35	10	25	50	450	100
211	—	10	15	15	35	500	100
RM 211	Amphotericin B	35	15	35	35	500	40
452	—	10	10	10	50	450	100
RM 452	Nystatin	10	10	10	50	450	100

* Minimal inhibitory concentration.

† Results are expressed as micrograms per milliliter.

subculturing in liquid medium, the increase in resistance was insignificant. There was a two- to threefold increase after 20 transfers.

Demonstration of cross-resistance on solid medium. The results for cross-resistance on solid medium are presented in Table 1. Higher concentrations of antibiotics were required to inhibit growth on agar medium than in liquid medium, and there was a definite cross resistance between candidin and amphotericin B. Strains resistant to candidin or amphotericin B did not show any significant increase in resistance to nystatin, fungimycin, or griseofulvin over the parent strains. On the other hand, strains showing relatively high resistance to candidin or amphotericin B in liquid medium were more sensitive to eulicin than the parent strains.

Alteration in virulence. The results for alteration in virulence are presented in Table 2 and 3. Strains that showed increased resistance to candidin or amphotericin B were less virulent for mice than the parent cells. To show that the decrease in virulence was not due to transferring the culture in vitro, an inoculum from the parent strain was transferred the same number of times as the resistant strain (25 transfers), but in the absence of the antibiotic. This strain, referred to in Table 2 as 452A, showed the same degree of virulence as the parent strain.

C. albicans RM 452 showed threefold increase

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

Strain no.	Exposed to antibiotic	Degree of resistance	No. of transfers	Culture dilution	Mean survival time	Survivors/total at 30 days	Survivors
					days		%
204	—	—	0	Undiluted	15	4/20	20
				1:5	20	10/20	50
				1:10	—	16/20	80
215	Candidin	150-fold	25	Undiluted	—	16/20	80
				1:5	—	20/20	100
				1:10	—	20/20	100
452	—	—	0	Undiluted	12	2/15	13.3
				1:5	19	6/15	40.0
				1:10	>30	10/15	66.6
216	Amphotericin B	4-fold	25	Undiluted	28	6/15	40.0
				1:5	>30	14/15	93.3
				1:10	>30	14/15	93.3
452A	—	—	25	Undiluted	13	2/15	13.3

TABLE 3. *Relation between resistance and virulence of Candida albicans*

Strain no.	Exposed to antibiotic	Degree of resistance	No. of transfers	Culture dilution	Mean survival time	Survivors/total at 30 days	Survivors
					days		%
452	—	—	0	Undiluted	5	0/20	0
				1:5	16	8/20	40
RM 452	Nystatin	3-fold	25	Undiluted	5	1/20	5
				1:5	13	6/20	30
211	—	—	0	Undiluted	3	0/20	0
				1:5	6	1/20	5
				1:10	8.5	0/20	0
RM 211	Amphotericin B	16-fold	30	Undiluted	>30	12/20	60
				1:5	>30	18/20	90
				1:10	—	20/20	100
211A	—	—	30	Undiluted	3	0/20	0

in resistance to nystatin, but did not show any alteration in virulence from the parent strain 452 (Table 3). On the other hand, the strain that developed 16-fold increase in resistance to amphotericin B showed a very marked decrease in virulence (Table 3). A control for the effect of transfer in culture was used (211A); it behaved like the parent strain.

Alterations in reactions characteristic of C. albicans strains. The following alterations in morphology were observed. Strain 215 (resistant to candidin) showed some differences from the parent strain 204. After 48 hr of growth in Sabouraud broth on a shaker, both strains grew exclusively in yeast form. After 7 days of incubation, infrequent pseudomycelia were seen in the case of 204, and more frequent ones were seen with 215. The difference, however, was not very significant. On Sabouraud agar, the gross appearance of both strains after 2 and 7 days of incubation was the same: white, creamy, glistening growth. On microscopic examination, both strains first grew in yeast form, and no pseudomycelia were seen in the case of strain 215 after 48 hr of incubation. After 7 days, cells of strain 204 grew in yeast form, and very few pseudomycelia were seen. The majority of cells of strain 215 grew in yeast form, but pseudomycelia were seen more frequently than in strain 204.

The growth of resistant strains RM 211, 216, and RM 452 did not show any differences in appearance from their parent strains 211 and 452 when examined microscopically. In the case of the strains that developed resistance to amphotericin B on gradient plates, differences in gross morphology were observed. The cells in liquid medium on a shaker grew as clumps that tended to settle to the bottom of the tube. On Sabouraud agar, the growth appeared granular. Microscopically, very frequent pseudomycelia were seen in cultures growing in liquid and on solid medium.

Chlamydospore formation. The resistant strains, whether developed in liquid or on solid medium, retained the ability to form chlamydospores on a suitable medium. The thick-walled, spherical, refractile structures formed also accumulated trypan blue and, thus, appeared blue.

Growth on Bi GGY Agar. The results for growth on Bi GGY Agar are shown in Fig. 4. Strain 215 (resistant to candidin) and strains 216 and RM 211 (resistant to amphotericin B) did not give the jet-black growth characteristic for *C. albicans* (Nickerson, 1953). Controls were used to demonstrate the effect of subculturing in vitro on the ability to reduce bismuth sulfite. No change was observed. In the case of the strains that showed a growth lighter in color than the parent strains, it was not clear whether that was due to less growth or decreased ability to reduce the bismuth complex. To answer this question, suitable samples of the parent and resistant strains were plated on Sabouraud agar and on Bi GGY Agar to give isolated colonies. All strains, except RM 211, grew equally well on Sabouraud agar and Bi GGY Agar with regard to the number of colonies produced. There were, however, differences in the size of colonies of the parent and resistant strains on Bi GGY Agar. The colonies of all the resistant strains used, except RM 452, were smaller than their corresponding parent strains after 60 hr of incubation, but the depth of the color of the colonies appeared similar. Strain RM 452 gave colonies identical to those of its parent strain 452. After 60 hr of incubation, plates of Bi GGY Agar with colonies of the parent strains were kept at 4 C. The resistant strains were allowed to continue growing at room temperature. Strain 216 required approximately 86 hr and strain 215 required 18 hr to give colonies similar in size and color to those of their parent strains.

The growth of strain RM 211 (16-fold resistant to amphotericin B), unlike that of other resistant strains in our study, yielded fewer colonies on Bi GGY medium than on Sabouraud medium. Some of the colonies of RM 211 that were able to grow on Bi GGY medium were transferred into Sabouraud broth and grown on a shaker. After reaching the stationary phase, their

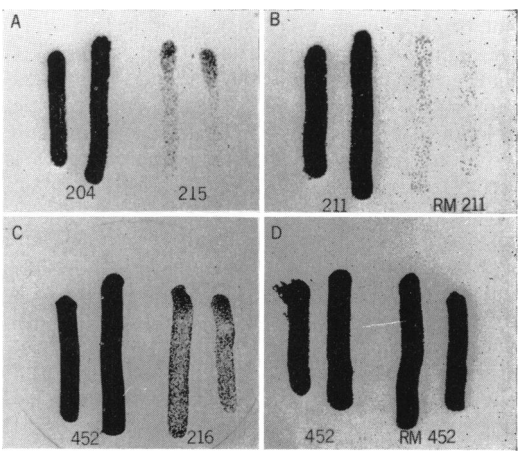

FIG. 4. *Growth of Candida albicans on Bi GGY Agar. (A) Strain 204, parent strain; strain 215, rendered resistant to candidin. (B) Strain 211, parent strain; strain RM 211, rendered resistant to amphotericin B. (C) Strain 452, parent strain; strain 216, rendered resistant to amphotericin B. (D) Strain 452, parent strain; strain RM 452, transferred in nystatin-containing medium.*

sensitivity to the bismuth complex was rechecked by comparing the number of cells on Sabouraud agar and Bi GGY medium. These cell suspensions were also checked for resistance to amphotericin B. It was found that the cell populations were not inhibited on Bi GGY medium, and that some of these cell populations were as sensitive to amphotericin as the parent strain.

DISCUSSION

Previous investigators have reported that high resistance to polyene antibiotics was not attained by *C. albicans* (Donovick et al., 1955; Littman, Pisano, and Lancaster, 1958; Stout and Pagano, 1956). In this study, three strains (204, 211, and 452) and three polyenes (candidin, amphotericin B, and mystatin) were used. The two techniques employed were subculturing in gradually increasing concentrations of an antibiotic in liquid medium on a shaker, and transferring on gradient plates. Axelrad (1960) showed that candidin did not diffuse readily through the agar medium. The present report showed that neither nystatin nor amphotericin B diffused well in Sabouraud agar medium prepared with Difco agar. However, when a purified agar, Ionagar no. 2 (Oxoid), was used in preparing the medium, both antibiotics diffused well, and resistance to amphotericin B was demonstrated. Development of resistance to nystatin was not shown by any of the strains used or any of the techniques employed. Only a two- to threefold increase in resistance, which might not be significant, was encountered in this study. Other investigators (Donovick et al., 1955; Littman et al., 1958; Stout and Pagano, 1956; Axelrad, 1960) were unable to demonstrate the development of resistance of *C. albicans* to nystatin.

Cross-resistance among polyene antibiotics is recognized. Sorenson, McNall, and Sternberg (1959) and Littman et al. (1958) recorded the interrelationship between amphotericin B and nystatin. 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. In our experiments, there was cross-resistance between candidin and amphotericin B. Although fungimycin is a heptaene, it did not show any cross-resistance to amphotericin B or candidin. There was no cross-resistance between the polyenes and the nonpolyenic antibiotics used in this study.

C. albicans strains that showed a significant resistance to candidin or amphotericin B were more sensitive to eulicin than their parent strains. The collateral sensitivity could not be explained on the basis of chemical structure. Strains of *C. albicans* resistant to candidin or amphotericin B were less virulent for mice than the parent sensitive strain. A strain that was only fourfold resistant to amphotericin B in broth showed a significant loss in virulence. These results agreed with the observation of Lones and Peacock (1959), who found the same relation with strains of *C. albicans* resistant to amphotericin B. Strains that were two- to threefold resistant to nystatin in liquid medium did not show any resistance on agar and did not change in virulence for mice.

Other changes in properties were observed. Strains resistant to amphotericin B or candidin were slower than their parent strains in forming the characteristic jet-black growth on Bi GGY medium. This medium contains bismuthyl polysulfite and has been found to be useful for the isolation and identification of species of *Candida* (Nickerson, 1953). The bismuth complex acts as an electron acceptor. The mechanism of reduction of bismuth sulfite into sulfide may be similar to that of the reduction of selenite into metallic selenium, as described by Nickerson and Falcone (1963). They found that the system for reduction of selenite requires a quinone, a thiol substance, a pyridine nucleotide, and an electron donor. The polyene-resistant strains described in this report have a lower rate of metabolism than their parent strains. This may lead to a slow flow of electrons, a slow rate of formation of reduced diphospho- or triphosphopyridine nucleotides, and a low level of —SH formation. These changes are expressed in a slow rate of growth, a tendency to grow in filamentous form, and a decreased ability to reduce bismuth sulfite. It was suggested (Nickerson, 1953) that the highly reducing material(s) secreted by *C. albicans* may play a role in the disease processes caused by this organism. In our investigation, all the resistant strains that were less able to reduce bismuth sulfite (and showed a slow rate of growth) demonstrated a significant loss in virulence.

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