Relationship Between Polyene Resistance and Sterol Compositions in *Cryptococcus neoformans*

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Six mutants of *Cryptococcus neoformans* resistant to nystatin and pimaricin and three mutants resistant to amphotericin B were isolated by ultraviolet irradiation techniques from two wild-type strains. The major sterols of the wild-type strains were Δ⁷-ergosten-3β-ol and ergosterol. All six mutants resistant to nystatin and pimaricin showed either loss of ergosterol and concurrent production of Δ⁷, 22-ergostadien-3β-ol and Δ⁷-ergosten-3β-ol, or loss of both the wild-type sterols, with production of Δ⁸(9)-ergosten-3β-ol and Δ⁸, 22-ergostatri-en-3β-ol. The mutants producing Δ⁷, 22-ergostadien-3β-ol and Δ⁷-ergosten-3β-ol showed relatively low levels of resistance to nystatin and pimaricin, whereas the mutants producing Δ⁸(9)-ergosten-3β-ol and Δ⁸, 22-ergostatrien-3β-ol showed a high level of resistance to either drug. Although highly resistant to amphotericin B, however, the three mutants produced sterol compositions identical to those of the wild types, indicating that the strains acquired resistance other than by alteration of the membrane sterols. The mutants producing Δ⁸(9) and Δ⁸, 22 sterols were not virulent for mice, showed reduced growth rates at 25 C, and failed to grow at 37 C. The other mutants showed a slightly reduced rate of growth both at 25 and 37 C, and the virulence in mice was slightly reduced in comparison with that of the wild types. These comparisons were on gross observations and were not statistically analyzed.

A number of authors (1, 2, 5–8, 10, 11, 14, 15) have suggested that a most likely mechanism by which fungi acquire polype resistance is through alterations of the sterols of the cell membrane.

Although isolation from clinical specimens of *Cryptococcus neoformans* highly resistant to the polype antibiotics has not been reported, a modest decrease in susceptibility to amphotericin B has been found to be induced in vivo during therapy (3). Induction of the resistance in vitro by exposing cells of *C. neoformans* to amphotericin B (3, 4) or to other polypes (4) has also been observed. Two of three nystatin-resistant mutants isolated by Bodenhoff (4) were cross-resistant to amphotericin B, whereas cross-resistance to nystatin was found in all three of the amphotericin B-resistant mutants. Sterol compositions in these mutants or in wild-type *C. neoformans* have, however, never been determined.

In the present paper, we report the sterol patterns of wild-type and ultraviolet (UV)-induced mutants of *C. neoformans* resistant to polypes. The growth pattern and the virulence of mutants for mice were also compared with those of wild types.

**MATERIALS AND METHODS**

**Strains.** Two strains of *C. neoformans*, 3737 and 3769, from clinical specimens were employed for the induction of mutants. These strains, which had not been exposed previously to the polype antibiotics either in vivo or in vitro, were maintained on malt extract agar medium at 25 C.

**Determination of minimal inhibitory concentration.** Cells from 48-h cultures of each strain were suspended in saline and about 1,000 cells per plate were spread on duplicate sets of polype media (see below) and incubated at 30 C for 72 h. Scoring for the growth was made as “+” for development of 50 or more colonies, “−” for total inhibition, and “±” for less than 49 colonies. If a sharp onset of total inhibition (−) is observed, then the lowest concentration of drug producing this is given in Table 1 as the minimal inhibitory concentration; otherwise, a range of concentrations covering partial inhibition (±) and the lowest concentration producing total inhibition is given.

The concentrations of the polype antibiotics used were as follows. Twofold dilutions of amphotericin B, from 32 to 0.25 µg/ml; nystatin, from 80 to 5 U/ml;
and pimaricin, from 250 to 0.625 and 50 μg/ml, respectively, were incorporated into malt extract agar medium. The sources of antibiotics and commercial ergosterol and the preparation and storage of stock solutions were as described in a previous paper (8).

**Isolation of mutants.** The cells from 48-h cultures of the strains 3737 and 3769 were suspended in saline, and the suspensions were then diluted to yield about 1 x 10^8 cells per ml. The cell suspensions (6 ml) were transferred into malt extract broth medium (150 ml) after UV irradiation from a 15 W germicidal lamp for 20 s. After a 72-h incubation at room temperature on a shaker, the broth cultures were plated on malt extract agar media containing the antibiotics. The colonies formed on the highest concentration of the antibiotics after a 5-day incubation at 30°C were transferred to a fresh drug medium of half the original concentration. The purification of the cultures was carried out by subculturing cells from a single colony formed on a drug medium. The cultural characteristics and size of the colonies of the mutants on malt extract agar medium at different temperatures were compared.

**Virulence for mice.** Female, general purpose (NIH), white mice weighing 19 to 21 g were infected by injection of 0.5 ml of cell suspensions in saline prepared from 72-h cultures of 3769 or from each of seven mutants derived from the strain. Two levels of inoculum, determined by hemocytometer and plate count as 2 × 10^5 and 4 × 10^6, were used. Five mice were injected with each inoculum intravenously and were observed daily up to 33 days for survival. On day 33 postinfection, surviving mice were sacrificed, and the brain was used to recover the strain as well as for the observation of in vivo morphology. Of the mice receiving the wild type, the last mouse (which died on day 18 postinfection) was used for this purpose.

**Sterol extraction.** The strains were cultured in malt extract broth medium for 5 days at 30°C on a gyrotyr shaker (115 rpm), and then the cells were harvested and treated with methanolic KOH for 2 h at reflux (8). The nonsaponifiables were extracted with hexane. After removal of the solvent, the crude hexane extracts (1 g) were subjected to adsorption on column chromatography on alumina (30 g). The fractions were eluted as follows: (i) petroleum ether-benzene (9:1, 100 ml; 1:1, 100 ml); (ii) benzene-ether (9:1, 100 ml; 3:1, 100 ml; 1:1, 100 ml); (iii) ether-methanol (19:1, 100 ml; 4:1, 100 ml); and (iv) ethyl acetate-chloroform-methanol-acetic acid (13:15:3:1, 100 ml). The sterols in each fraction were recovered by evaporating the solvents and detected by subsequent thin-layer chromatography. Most of the sterols were usually eluted with benzene containing 10 and 25% ether. The sterol mixtures were then chromatographed on 5% AgNO₃-impregnated Silica Gel G thin-layer plates with benzene-ethyl ether (5:1) as the developing solvent. The separated components were detected with the Liebermann-Burchard reagent (8) and scraped from the plates. The sterols were then eluted with chloroform and recovered by evaporation of the solvent at room temperature in a stream of nitrogen.

The UV spectrophotometry of the sterols was carried out by scanning between 200 and 310 nm on methanolic solutions with a Cary model 14 spectrophotometer.

Gas-liquid chromatography was carried out with a Glowa glass chromatograph containing a column (72 by 0.25 in. [outer diameter]) packed with 1% OV-17 on Supelcoport (Supelco, Inc., Bellefonte, Pa.). This column was used isothermally at 230°C with a flame ionization detector. Gas chromatography-mass spectrometry was effected with an LKB-9000 with a column (72 by 0.25 in. [outer diameter]) containing 1% OV-17 on Supelcoport. This column was programmed from 230°C at 6°C per min. Mass spectra were measured at an ionizing voltage of 70 eV, a source temperature of 270°C, and a scan speed of approximately 10 s per decade in mass.

**RESULTS**

Seven mutant strains resistant to the polyene antibiotics were isolated from a UV-irradiated population of wild-type strain 3769. Three mutants obtained on amphotericin B medium (8 μg/ml) were designated as CNAR1, CNAR2, and CNAR3. One mutant grown on nystatin medium (20 U/ml) was designated as CNNR1, and three mutants obtained on the pimaricin medium (5 μg/ml) were designated as CNPR1, CNPR2, and CNPR3. Two mutants, CNPR4 and CNPR5, were isolated from the UV-irradiated cultures of strain 3737 grown on the pimaricin medium (2.5 μg/ml).

The susceptibility tests against the polyene antibiotics after the purification of the mutants showed that the resistance of the mutants was reproducible with respect to the drug from which they had been isolated, but the levels of resistance and the cross resistance patterns varied widely (Table 1). The minimal inhibitory concentration for amphotericin B in the mutants increased from 32- to 64-fold in CNAR2 and from 64- to 128-fold in CNAR1 and CNAR3 as compared with the wild type. These mutants remained susceptible to nystatin and...
pimaricin, although one mutant, CNAR3, showed slightly increased resistance to nystatin. One mutant, CNNR1, showed only a two-fold increase of minimal inhibitory concentration for nystatin compared with the wild types and was cross-resistant to pimaricin but not to amphotericin B.

Two of the five pimaricin-resistant mutants (CNPR4 and CNPR5) showed an eightfold increase of minimal inhibitory concentration of pimaricin and the remaining three (CNPR1, CNPR2 and CNPR3) showed a 16- to 40-fold increase. These mutants were cross-resistant to nystatin but not to amphotericin B, with the exception of CNPR2, which showed a two- to fourfold increase of minimal inhibitory concentration for amphotericin B.

Although varied, the growth rate of each of the mutants was generally slower than that of the corresponding wild type. Of nine mutants, six (CNAR1, CNAR2, CNAR3, CNNR1, CNPR4, and CNPR5) showed a fair growth rate at 25 and 37°C, while the remaining three (CNPR1, CNPR2, and CNPR3) showed drastically reduced growth rates at 25°C and failed to grow at all at 37°C.

The colonies of CNPR1, CNPR2, and CNPR3 were less mucoid than those of CNAR1, CNAR3, CNNR1, CNPR4, and CNPR5 or those of the wild types. Colonies of six strains, CNNR1 and CNPR1 to 5, produced a light orange pigment on malt extract agar or Sabouraud dextrose agar. Capsules were also markedly reduced in size in strains CNPR1, CNPR2, and CNPR3, but were similar to those of the wild types in mutants CNAR1, CNAR3, CNNR1, CNPR4, and CNPR5 (Fig. 1).

The UV spectrum of the mixture of sterols isolated from the wild types showed peaks at 271, 282, and 293 nm (Fig. 2B) typical of ergosterol (Fig. 2A) and a larger peak at 205 nm. Sterols from three amphotericin B-resistant mutants, CNAR1, CNAR2, and CNAR3, showed UV absorption spectra identical to the wild type, though the maxima assigned to ergosterol were slightly less intense than in the wild types. The remaining six mutants showed one peak at 205 nm (Fig. 2C) but no peaks between 220 and 310 nm, indicating the absence of ergosterol.

The ergosterol of the wild types and amphotericin B-resistant mutants was readily separated from the other sterols by thin-layer chromatography. Six other mutants showed only a single spot on thin-layer chromatography. The analysis of the sterols by gas-liquid chromatography showed further differences among the mutants. A comparison of the gas-liquid chromatograms of the sterol mixtures revealed three types of patterns. The first type, of which CNAR3 is representative (Fig. 3), was, apart from the relatively low proportion of ergosterol, the same as the wild types. The minor peak 1 was confirmed by gas chromatography-mass spectrometry to be due to ergosterol, and the mass spectrum of the major peak 2 (Fig. 4) was identical to that of authentic \( \Delta^7 \)-ergosten-3\( \beta \)-ol. Similar results were given by the amphotericin B-resistant mutants, CNAR1 and CNAR2. The wild types also belong to this class, and thus it could be concluded that the resistance of these mutants to amphotericin B clearly was not related to the sterol composition of the cells.

The second type of gas-liquid chromatography exemplified by CNPR4 (Fig. 3) includes CNNR1, CNPR4, and CNPR5. The gas chromatography-mass spectrometry analysis revealed that the mass spectrum of the major peak 1 was identical with that of \( \Delta^7 \)-22-ergostadien-3\( \beta \)-ol, and that of the minor peak 2 with that of \( \Delta^7 \)-ergosten-3\( \beta \)-ol (Fig. 4). The authentic samples of these compounds were found to be inseparable by gas-liquid chromatography from the corresponding sterols of the mutants. The desaturation of \( \Delta^4 \)-C\( \alpha \) in the biosynthesis of ergosterol seems to have been blocked in these mutants. The relative proportion of peak 2 varied slightly between mutants and was reduced as compared with wild types.

The third type of gas-liquid chromatography, shown by CNPR2 in Fig. 3, was given by CNPR1, CNPR2, and CNPR3. The relative proportion of peak 1, a minor component, was usually higher in CNPR2 than in the other two strains. The mass spectrum of peak 1 (Fig. 5) revealed its molecular weight to be 396, which would thus permit its formulation as an ergostatrienol. This spectrum was identical with that of \( \Delta^5 \)-, \( \Delta^9 \)-, and \( \Delta^{12} \)-ergostatrien-3\( \beta \)-ol, which has previously been isolated from an alga (12) and a fungus (9). The mass spectrum of compound 2, the major sterol of these mutants, showed its molecular weight to be 400 (Fig. 5), but was significantly different from the mass spectrum of \( \Delta^7 \)-ergosten-3\( \beta \)-ol. This new mass spectrum proved to be identical with that of synthetic \( \Delta^8 \)-(9)-ergosten-3\( \beta \)-ol. These mutants have therefore apparently lost the enzyme system responsible for the isomerization of the \( \Delta^8 \)-(9) system to a \( \Delta^7 \) system. Sterol compositions of the wild types and of the mutants are summarized in Table 2.

The results of mouse lethality studies conducted on seven mutants and one wild type are shown in Table 3. A fatal infection could not be
Fig. 1. Wild type and mutants of C. neoformans in vitro (India ink preparations, A, C, and E) and in vivo (mouse brain, B, D, and F). (A) Indian ink preparation of wild-type strain 3769 grown on malt extract agar, ×1200. (B) The same strain seen in squash preparation of mouse brain, ×1200. (C) The mutant strain CNNR1 in vitro, ×1200. (D) The strain CNRR1 in vivo, ×1200. (E) The mutant strain CNPR2 with diminished capsule in vitro, ×1200. (F) The same strain in vivo, ×1200.

Produced with the four mutant strains, CNAR1, CNPR1, CNPR2, and CNPR3, despite several attempts. The mice infected with these strains appeared well throughout the observation period, and squash preparations and cultures of brain at day 33 postinoculation revealed no
C. neoformans. The strain CNPR1 was again inoculated intravenously into 10 mice, and the mice were sacrificed at 1-day intervals to recover the strain. In mice sacrificed after day 6, no C. neoformans cells were recovered by culture or observed microscopically in squash preparations of the lung and brain. The cells seen in the squash preparation of the brain tissue from mice 72 h after inoculation were devoid of capsules. Whereas the remaining three mutants, CNAR2, CNAR3, and CNNR1, produced fatal infections in mice, they were definitely less virulent than the wild type. Two of the most virulent mutants, CNAR2 and CNNR1 (4 × 10^8 cells/mouse), killed only one each of five mice after 20 days of inoculation, while the wild type killed all 5 mice within 18 days postinoculation. Squash preparation of brains of the mice infected with CNAR2, CNAR3, and CNNR1 revealed well-capsulated C. neoformans yeasts equivalent to those in mice infected with the wild type (Fig. 1).

**Fig. 2.** UV spectra of ergosterol (A) and the sterols (B and C) from wild-type and polyene-resistant mutants of C. neoformans.

**Fig. 3.** Gas-liquid chromatograms of the sterols from wild-type and polyene-resistant mutants of C. neoformans.
DISCUSSION

It is noteworthy that the principal sterol of wild-type *C. neoformans* is Δ⁷-ergosten-3β-ol rather than ergosterol as is found in other yeasts or filamentous fungi (1, 2, 5, 6, 8, 10, 11, 13–15). Ergosterol is present in *C. neoformans* as a minor component. Since other investigators (1, 2, 5, 6, 10, 11, 15) did not use malt extract broth, one might suspect that the difference can be
Table 2. Major sterols of wild type and mutants of C. neoformans resistant to polyenes

<table>
<thead>
<tr>
<th>Strains</th>
<th>Drug medium used for the isolation</th>
<th>Major sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild types</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNAR1</td>
<td>Amphotericin B (8 µg/ml)</td>
<td>( \Delta^7 )-ergosten-3(\beta)-ol and ergosterol (( \Delta^5 ), ( \Delta^7 ), 22-ergostatrien-3(\beta)-ol)</td>
</tr>
<tr>
<td>CNAR2</td>
<td>Amphotericin B (8 µg/ml)</td>
<td>( \Delta^7 )-ergosten-3(\beta)-ol and ergosterol</td>
</tr>
<tr>
<td>CNAR3</td>
<td>Amphotericin B (8 µg/ml)</td>
<td>( \Delta^7 )-ergosten-3(\beta)-ol and ergosterol</td>
</tr>
<tr>
<td>CNNR1</td>
<td>Nystatin (20 U/ml)</td>
<td>( \Delta^7 ), 22-ergostadien-3(\beta)-ol and ( \Delta^7 )-ergosten-3(\beta)-ol</td>
</tr>
<tr>
<td>CNPR1</td>
<td>Pimaricin (5 µg/ml)</td>
<td>( \Delta^6 ), 8(9)-, 22-ergostatrien-3(\beta)-ol</td>
</tr>
<tr>
<td>CNPR2</td>
<td>Pimaricin (5 µg/ml)</td>
<td>( \Delta^6 )-ergosten-3(\beta)-ol and ( \Delta^6 ), 8(9), 22-ergostatrien-3(\beta)-ol</td>
</tr>
<tr>
<td>CNPR3</td>
<td>Pimaricin (5 µg/ml)</td>
<td>( \Delta^6 )-ergosten-3(\beta)-ol and ( \Delta^6 ), 8(9), 22-ergostatrien-3(\beta)-ol</td>
</tr>
<tr>
<td>CNPR4</td>
<td>Pimaricin (2.5 µg/ml)</td>
<td>( \Delta^7 )-ergosten-3(\beta)-ol and ( \Delta^6 ), 8(9), 22-ergostatrien-3(\beta)-ol</td>
</tr>
<tr>
<td>CNPR5</td>
<td>Pimaricin (2.5 µg/ml)</td>
<td>( \Delta^6 )-ergosten-3(\beta)-ol and ( \Delta^6 ), 8(9), 22-ergostatrien-3(\beta)-ol</td>
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Table 3. Number of survivors on each day in mice infected with wild type, 3769, and six mutant strains of C. neoformans resistant to polyene antibiotics

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Strain</th>
<th>No. of survivors on day:</th>
<th>% Survival at 33 days</th>
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<tr>
<td></td>
<td></td>
<td>1 to 5</td>
<td>6</td>
</tr>
<tr>
<td>4 \times 10^4</td>
<td>CNAR1</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>CNAR2</td>
<td>5</td>
<td>5</td>
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<tr>
<td></td>
<td>CNAR3</td>
<td>5</td>
<td>5</td>
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<td></td>
<td>CNNR1</td>
<td>5</td>
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<td>CNPR1</td>
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<td></td>
<td>CNPR2</td>
<td>5</td>
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</tr>
<tr>
<td></td>
<td>CNPR3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2 \times 10^7</td>
<td>3769</td>
<td>4</td>
<td>4</td>
</tr>
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attributed to the media compositions. Our tests with *Saccharomyces cerevisiae* and *Aspergillus fennelliae* (8), grown under the identical environmental conditions as for *C. neoformans*, still showed ergosterol to be the major sterol in these two organisms. The development of polyene resistance with concurrent alterations of sterol compositions was also reproducible in *C. neoformans*, specifically in the mutants isolated from nystatin and pimaricin media after UV irradiation. Ergosterol was completely lost in these mutants which accumulated its biosynthetic precursors such as \( \Delta^7, 22 \)-ergostadien-3\(\beta\)-ol or \( \Delta^5, 8(9), 22 \)-ergostatrien-3\(\beta\)-ol. The susceptibility of the wild type to polyenes, therefore, appears to be due to the presence of ergosterol rather than \( \Delta^7 \)-ergosten-3\(\beta\)-ol. The loss of the C5—C6 double bond in the sterols in the mutants CNNR1, CNPR4, and CNPR5 accompanied twofold and eightfold increases in resistance to nystatin and pimaricin, respectively, but the susceptibility to amphotericin B was undiminished. These results indicate that \( \Delta^7, 22 \)-ergostadien-3\(\beta\)-ol binds less effectively with nystatin and pimaricin but as effectively as ergosterol with amphotericin B. The mutants such as CNPR1, CNPR2, and CNPR3 showed a metabolic block for the \( \Delta^6 \) \(-\Delta^7 \) isomerization, accumulating \( \Delta^6(9)- \)ergosten-3\(\beta\)-ol and \( \Delta^6, 8(9), 22 \)-ergostatrien-3\(\beta\)-ol. These strains showed higher resistance to nystatin and pimaricin as compared with the mutants producing \( \Delta^7, 22 \)- and \( \Delta^7 \)-sterols.

Resistance to amphotericin B, though low, was also detected in the mutants producing \( \Delta^6(9) \)- and \( \Delta^6, 8(9), 22 \)-sterols. These results agree with the findings in the mutants of *S. cerevisiae* (5) in which the effectiveness of polyene bindings was in the following order: \( \Delta^5, 7 \)-, \( \Delta^7 \)- and \( \Delta^8 \)-sterols.
The development of polyene resistance in fungi, however, does not appear to be totally dependent on the sterol changes of the cells. The mutants resistant to amphotericin B showed no substantial changes in sterol composition. Only a slight decrease in the amount of ergosterol as compared with the wild types was seen. The reduced amount of ergosterol in the mutants is not the immediate cause of resistance since the mutants lacking ergosterol are still sensitive to amphotericin B. The resistance of these mutants appears to be specific to amphotericin B since they remained sensitive to nystatin and pimaricin. One of the probable mechanisms related to this phenomenon may be an impairment of permeability to amphotericin B. Such a possibility is currently being investigated. The growth rate of mutants in vitro seemed to be related to their sterol patterns. Thus, the mutants produced the same sterol compositions as the wild types (i.e., CNAR1, CNAR2, and CNAR3) and showed near wild-type growth at 25 C and 37 C. The virulence of the strains, however, could not be assessed by their in vitro growth rate alone except in the cases of the \( \Delta^7,22 \) and \( \Delta^5,8,9,22 \) sterol producers. The strains CNAR2 and CNAR3, though much less virulent than the wild type, produced fatal infections in mice. The strain CNAR1, however, was avirulent and disappeared from mouse brain and lung within 33 days of inoculation. The mutants producing \( \Delta^7,22 \)-ergostadien-3\( \beta \)-ol and \( \Delta^1 \)-ergosten-3\( \beta \)-ol showed a reduced rate of growth compared with that of the wild type. Although less virulent than the wild type, one of the strains tested (CNNR1) was the most virulent among the mutants. Reverse mutations of some of the population during the course of infection in mouse tissue cannot be ruled out in this case. In fact, when cultured consecutively on drug-free media, reverse mutation occurs more readily in this strain than in others. The strains producing the \( \Delta^8,9 \) and \( \Delta^5,8,9,22 \)-sterols showed no growth in vitro at 37 C and a drastically reduced rate of growth at 25 C. Accordingly, these strains were avirulent for mice and disappeared from mouse tissue 6 days after inoculation.

It is unlikely that such strains producing \( \Delta^8,9 \) and \( \Delta^5,8,9,22 \)-sterols can be isolated from patients during clinical application of the polyene antibiotics for cryptococcosis. However, the possibility of isolating mutants such as CNAR2 and CNAR3 or the mutants producing \( \Delta^7,22 \) and \( \Delta^5 \)-sterols cannot be excluded since they retain some pathogenicity.

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**LITERATURE CITED**


