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NOTES

Polyoxin D Inhibits Growth of Zoopathogenic Fungi

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We demonstrated that polyoxin D at millimolar concentrations caused marked morphological alterations of the human pathogens *Candida albicans* and *Cryptococcus neoformans*. *C. albicans* incubated in the presence of this drug grew in long chains that were severely swollen. Polyoxin D inhibited the growth of *C. neoformans* and killed cells of both the yeast and the hyphal phase of *C. albicans*. These observations give the first evidence that polyoxin antibiotics can kill zoopathogenic fungi.

Fungal infections are a common complication in patients treated with immunosuppressants, cytotoxic drugs, antibiotics, and corticosteroids (9). The decision concerning initiation of therapy for fungal infections poses a major problem for the attending physician; the number of effective antifungal drugs is small, and often these drugs possess marked toxicity to the host (2, 16, 25). Although ketoconazole has been suggested as a rather nontoxic drug for general use in systemic fungal infections, a complete clinical evaluation is not available (10, 23). It is clear that specific and nontoxic antifungal drugs are needed.

Polyoxins (6, 13, 14) are peptidyl nucleosides that inhibit the synthesis of chitin in fungal cell walls and insect exoskeletons and are effective antibiotics against phytopathogenic fungi. Because they are not toxic to vertebrates and there is no detectable toxicity in mammalian systems, these antibiotics would be logical candidates for use as agents against systemic fungal infections. However, both natural and synthetic analogs of polyoxin D have been reported consistently to be ineffective against zoopathogenic fungi (5, 6, 8, 12–14, 20, 22). We chose to investigate this problem in more detail and report here that polyoxin D itself markedly affects the morphology of *Candida albicans* and *Cryptococcus neoformans* and is fungicidal to *C. albicans* at millimolar concentrations.

Polyoxin D was purified (21) from crude polyoxin and was also obtained from Sigma Chemical Co., St. Louis, Mo. In vitro activity of chitin synthetase was assayed by using a mixed membrane fraction (5) from *C. albicans* H-317, a clinical isolate obtained from the Centers for Disease Control, Atlanta, Ga. Chitin synthetase was activated by incubation with trypsin, and enzyme activity was measured by incorporation of radioactivity from UDP-*N*-acetyl-[^{14}C]glucosamine (Amersham Corp., Arlington Heights, Ill.) into chitin. An 80% inhibition of incorporation of label into chitin was seen at a 10^{-5} M concentration of polyoxin D. This is the first report of the inhibition of *C. albicans* chitin synthetase by polyoxin D. The activity found is similar to the amount of polyoxin D required to inhibit effectively the chitin synthetase from *S. cerevisiae* (15) and filamentous fungi (1, 7, 11, 20).

Polyoxin D has been previously reported to affect the morphology of several fungi, including *Saccharomyces cerevisiae* (3) and *Cochliobolus miyabeanus* (6). We found that at millimolar concentrations of this antibiotic, normal budding of *S. cerevisiae* was inhibited and, as a result, structures resembling chains of cells were seen. Occasionally, a pair of cells was observed with an extrusion of cytoplasmic material at their interface, an effect noted previously (3). It is known that polyoxin D inhibits chitin synthetase of *S. cerevisiae* (15), and chitin is known to be involved as an important component of the primary septum of this yeast (4). Thus, we conclude that the chaining which we observe at high drug concentrations is the result of the inhibition of septum formation between the mother and daughter cells. Furthermore, we observed effects of polyoxin D on *Cochliobolus miyabeanus* similar to those reported (6).

We observed that polyoxin D, when incubated with *C. albicans*, caused extensive chaining (Fig. 1B). Bulbous cells were present usually at the end of the *C. albicans* chain and were sensitive to changes in the osmotic environment

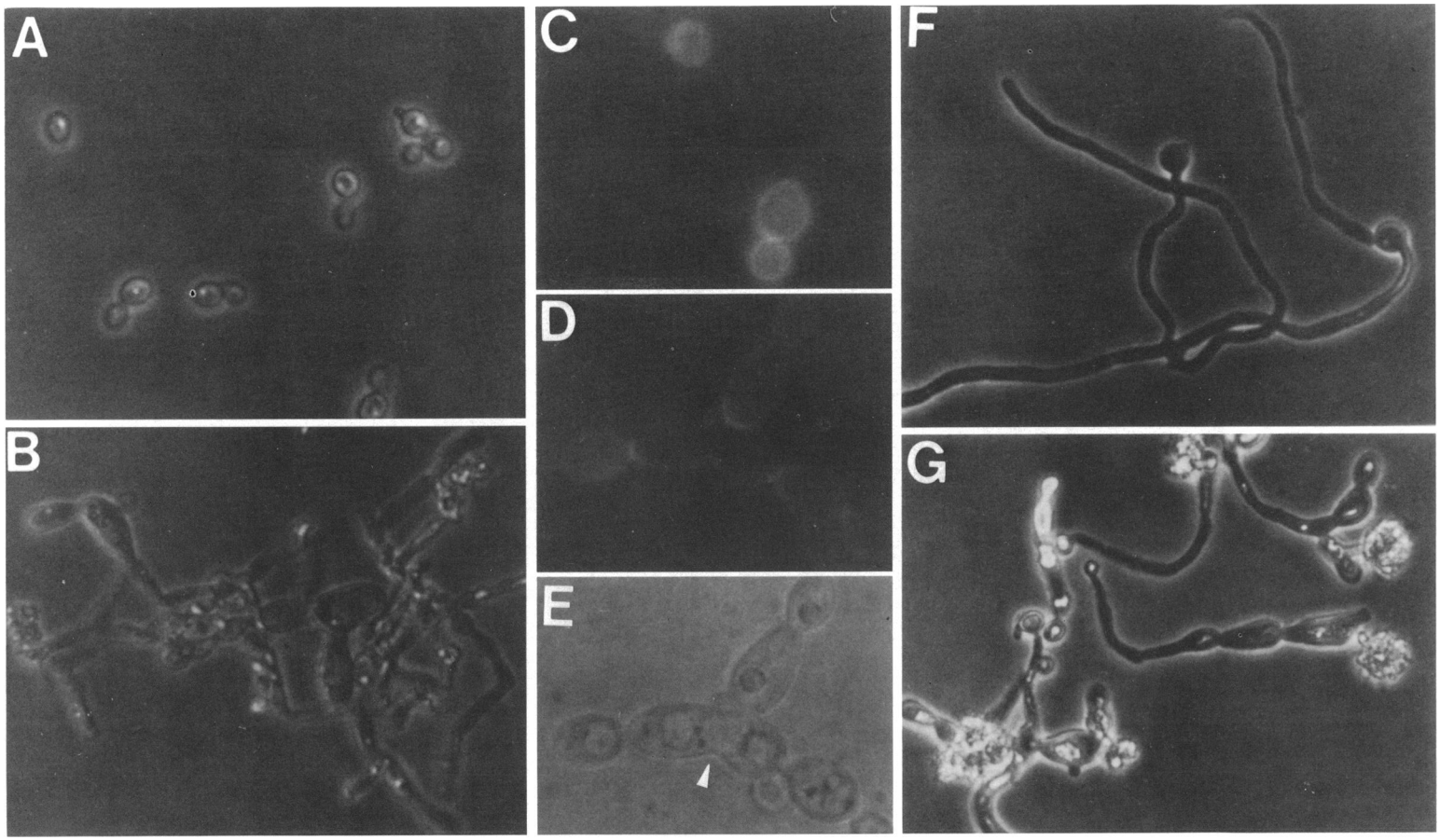

FIG. 1. Photomicrographs of *Candida albicans* incubated with and without polyoxin D. (A) *C. albicans* H-317 was grown in 0.17% yeast nitrogen base (Difco Laboratories, Detroit, Mich.) with 5% glucose and 0.66% isoleucine as a nitrogen source for 41 h at 37°C (magnification, ×490). (B) Same conditions as in (A) except incubation for 17 h with the addition of 8 mM polyoxin D (magnification, ×490). (C) Control culture grown as in (A) incubated with 0.01% calcofluor white M2R (Polysciences Inc., Warrington, Pa.) in phosphate-buffered saline at 37°C for 5 min. The cells were visualized by fluorescence microscopy (BG 3 filter and K510 suppression filter; Leitz Orthoplan) (magnification, ×640). (D) Same as (C) except cells incubated with 4 mM polyoxin D for 118 h (magnification, ×640). (E) Phase-contrast micrograph of same cells shown in (D) (magnification, ×640). The arrow indicates area where septum should be. (F) Cells were induced to form hyphae by changing the incubation conditions from pH 4.5 to pH 6.5 and incubated for 9 h (magnification, ×490). (G) Cells were induced as in (F). After a 3-h incubation at pH 6.5, they were incubated for 6 h more in the presence of 8 mM polyoxin D (magnification, ×490).

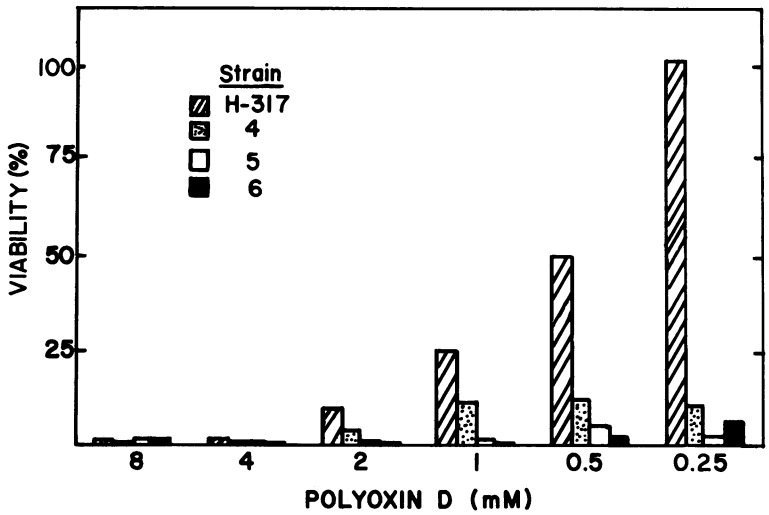

FIG. 2. Effect of polyoxin D on the viability of various *C. albicans* strains. Cells were grown under same conditions as described in the legend to Fig. 1A for 20 h to mid-logarithmic phase of growth. Portions of this inoculum were diluted to ca. 10^6 cells per ml in the same medium, and polyoxin D was added to the final concentrations as indicated. Portions of the cultures were removed at 2 days, diluted serially, and plated on potato dextrose agar with 0.5% yeast extract. The percent viability was taken to be the number of colonies determined by plate counts divided by the number of potential CFU determined by hemacytometer count, multiplied by 100.

of the incubation medium. When placed in water after treatment with polyoxin D, some cells burst, whereas in 1.5 M KCl, swollen cells plasmolyzed. Occasionally, the bulbous cells exploded upon continued incubation in the presence of greater than 2 mM polyoxin D. We believe that both formation of chains and the osmotic sensitivity of the yeast cells can be accounted for by the inhibition of chitin synthesis in the growing cells. Apparently, insufficient chitin levels lead to a weakened cell wall, resulting in the formation of cells which are swollen to two to three times their normal size.

To study further the inhibition of chitin synthesis, we stained control and polyoxin-treated cells with Calcofluor White-MZR (Polysciences Inc., Warrington, Pa.). This fluorescent dye complexes with chitin and other cell wall polysaccharides. It is clear that control cells (Fig. 1C) exhibit much more fluorescence than treated cells (Fig. 1D). It is striking that in some cases, no fluorescence occurs at the junction between two cells (Fig. 1D), whereas fluorescence is most intense at the septum for the control cells. As chitin has been shown to be localized to a great extent in the septum of *C. albicans* (24), we interpret these data to show that chitin synthesis is inhibited in these polyoxin D-treated cells. This conclusion was supported by phase-contrast microscopy (Fig. 1E), which clearly showed a vacuole extending across the septal region (arrow), and experiments with Primulin (Fluka, Switzerland), a chitin-specific fluorescent dye, that demonstrate no septa in polyoxin D-treated cells of the yeast or mold phases (data not shown). Thus, the inhibition of chitin synthesis in the presence of polyoxin D affects the ability of the cells to successfully complete the bud, and the cell continues to grow at the tip and forms chains of bulbous cells.

When polyoxin D was incubated with *C. albicans* (*C. albicans* H317 and three clinical isolates from St. Mary's Hospital, Knoxville, Tenn.), cell death occurred (Fig. 2). Marked differences in susceptibility to polyoxin D were noted. For three of the four strains examined, 90% or greater killing was observed with 0.5 mM polyoxin D. Polyoxin D also affected the growth of the mold phase of *C. albicans* (Fig. 1F and G). When polyoxin D was added to cultures at the time of germ tube induction, the germ tube was not formed. Rather, the morphology of the cellular extension from the mother cell was the same as that of yeast cells treated with polyoxin D. When polyoxin D was added 3 h after induction of germ tubes (Fig. 1F), the germ tube growth changed to the morphology of yeast cells treated with polyoxin D.

The effects of 1 mM polyoxin D on *C. albicans* could be prevented by incubation of the cells in the presence of 1% tryptone. Yeast extract (1%) or peptone (1%) protected the cells to a lesser extent. Previous evidence has shown a similar

prevention of polyoxin D effects on fungi (3, 17). We conclude that polyoxin D may be entering *C. albicans* through a peptide transport system. Thus, if a polyoxin analog which has higher affinity for the peptide transport system can be developed, it may cause death of *C. albicans* at micromolar concentrations.

We observed a marked effect of polyoxin D on the morphology and growth of *Cryptococcus neoformans* (NIH 3502) at concentrations above 0.5 mM. In the presence of inhibitor, cells were swollen to several times their normal size and cell debris was observed microscopically. Growth on minimal medium agar was significantly inhibited. After a 3- to 5-day incubation with 2 mM polyoxin D, no colonies were visible, whereas in control cultures colonies were readily visible with the naked eye. Inoculation of 10^6 cells per ml into liquid medium containing 2 mM polyoxin D resulted in a 99.9% growth inhibition compared with that of control cultures. Viability studies suggest that polyoxin D is fungistatic to *C. neoformans*.

The effects of fungicides and insecticides on chitin synthesis have received increasing interest; chitin is a most attractive target for antimycotic agents since this polymer is restricted in its biological distribution to fungi and insects (18, 19). In addition, studies demonstrated that polyoxin D is not toxic to various vertebrates (6, 13, 14). Our results are the first evidence that polyoxin D can inhibit zoopathogenic yeasts and point out a need for further investigation of the possibility of using chitin synthetase inhibitors as antifungal drugs.

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