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Antifungal Properties of Polymyxin B and Its Potentiation of Tetracycline as an Antifungal Agent

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High concentrations of polymyxin B inhibited the growth of *Candida albicans* and *Saccharomyces cerevisiae*. When these yeasts were incubated with concentrations of polymyxin B too low to affect growth, and were then exposed to tetracycline, protein synthesis was inhibited and at least 99% of the organisms were killed. Neither inhibition of protein synthesis nor cell death occurred in cultures treated with high concentrations of tetracycline alone. We conclude that polymyxin B at high concentrations affects the cell membrane of yeasts, which results in inhibition of growth. At low concentrations, it increases the permeability of the yeast cell membrane to tetracycline, which then inhibits protein synthesis and leads to cell death.

Polymyxin B is a surface-active bactericidal antibiotic which alters the permeability of the bacterial cell envelope by binding to the negatively charged phospholipid component of the membrane and causing cell lysis (4). Because eukaryotic cells also contain phospholipids in their cell membranes (7), it was reasonable to expect that polymyxin B would disrupt permeability barriers in them as well. Two previous studies have shown this to be true in a protozoan (6) and in the yeast *Candida tropicalis* (5). The latter study, however, failed to demonstrate a polymyxin B effect in a variety of other yeasts.

Several agents have been shown to be ineffective against yeasts because of failure to penetrate the cytoplasmic membrane and gain access to their site of action. For example, tetracycline has been shown to inhibit protein synthesis in extracts of yeasts (1) but was ineffective against whole organisms (C. N. Kwan et al., Bacteriol. Proc., p. 179, 1972). In this report, we have investigated the antifungal properties of polymyxin B against certain yeasts, and have exploited the changes in membrane permeability caused by this antibiotic to potentiate the antifungal effect of tetracycline.

MATERIALS AND METHODS

Organisms. *Saccharomyces cerevisiae* MG456 and *C. albicans* 374 are part of the standard stock-culture collection in our laboratory. They were maintained on Sabouraud dextrose agar (4% glucose, 1% peptone, and 1.5% agar) and were transferred at biweekly intervals to fresh culture media.

Chemicals and antibiotics. Polymyxin B sulfate (Aerosporin) was supplied by Burroughs-Wellcome Co. Tetracycline HCl was suppled by Lederle Laboratories. Both drugs were dissolved in sterile distilled water and were then added to the incubating cultures. [^{3}H]-leucine (40 Ci/mmole) and [^{14}C]-uracil (52 mCi/mmole) were obtained from Schwarz BioResearch, Inc.

Synergism studies. The susceptibility of each organism to polymyxin B and tetracycline was determined by the broth-dilution method, as previously described (2). A wide range of antibiotic concentrations was examined for each organism, and cell viability was determined by colony counts after 1, 2, 3, and 7 days of incubation. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of drug required to inhibit completely the growth of the organism over the designated time interval.

Synergism was also determined by colony counts of each organism in the presence of the antibiotics used singly and in combination. Exposure to the two drugs was done either by 24-hr pretreatment of the yeasts with polymyxin B or by simultaneous addition of the polymyxin B and tetracycline. Our definition of antifungal drug synergy was a decrease of 100-fold or more in colony counts caused by the drugs in combination as compared with the counts when the drugs were used singly (3). The level of each antibiotic used in the combined drug studies was well below the respective MIC. In the MIC and synergism studies, a standard inoculum of 5×10^4 to 10×10^4 cells/ml was incubated at 24 to 26 C.

Determination of RNA and protein synthesis. Ribo-

nucleic acid (RNA) synthesis and protein synthesis were determined by incubation of the yeasts in [^{3}H]-leucine and [^{14}C]-uracil in the presence of polymyxin B or tetracycline, or both. In some of the incubations, the yeasts exposed to both drugs were pretreated overnight with polymyxin B before the tetracycline was added. Portions of 1 ml of the cultures were precipitated with 1 ml of 10% trichloroacetic acid, filtered, and counted.

Results

Both *C. albicans* 374 and *S. cerevisiae* MG456 were susceptible to relatively low concentrations of polymyxin B (Fig. 1). The MIC values for the two organisms at each time interval differed, and increased as the incubation time was prolonged. Figures 2A and B illustrate the effects of polymyxin B on RNA and protein synthesis in both yeasts. Concentrations of polymyxin B below the MIC had little or no effect on either [^{3}H]-leucine or [^{14}C]-uracil incorporation. When concentrations of polymyxin B greater than the MIC were added to the cultures, both RNA and protein synthesis fell concomitantly.

Synergy was demonstrated by a decrease of colony counts in both yeasts with a combination of drugs at concentrations which exhibited little or no antifungal activity when employed singly (Fig. 3A and B). Tetracycline alone had no effect on growth of either *S. cerevisiae* MG456 or *C. albicans* 374 at concentrations as high as 500 μg/ml. For polymyxin B, the concentrations used in combination with tetracycline were less than half the MIC. For *C. albicans*, the MIC at 3 days was 30 μg/ml; for *S. cerevisiae*, it was 6.0 μg/ml (Fig. 1). Figures 4A and B show dose-response curves based on incorporation of [^{3}H]-leucine into protein with tetracycline alone and in the presence of various concentrations of polymyxin B. As with the colony count results, tetracycline and polymyxin B alone, at the concentrations used, had little effect. In combination, a dose response for each drug was demonstrated for protein synthesis, whereas [^{14}C]-uracil incorporation was not affected. In determinations of both the colony count and the incorporation of radioactive label, potentiation of tetracycline occurred only when the yeasts were first incubated in the polymyxin B alone overnight. When the two antibiotics were added simultaneously, or the tetracycline was added first, antagonism resulted and the antifungal effects of polymyxin B were reversed by tetracycline (data not shown).

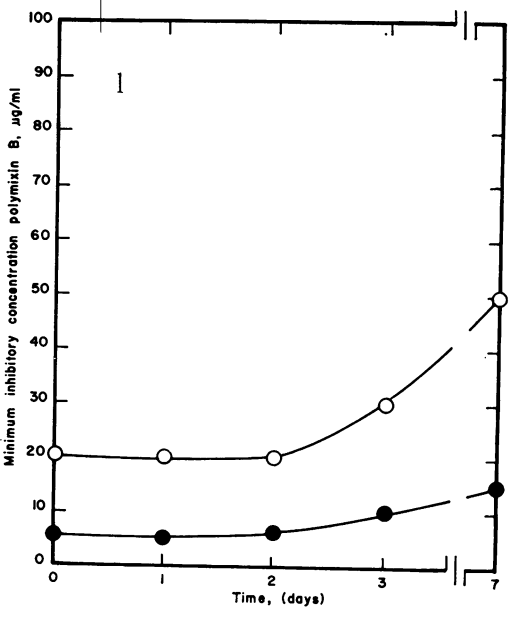

FIG. 1. *Minimal inhibitory concentrations of polymyxin B for C. albicans and S. cerevisiae at various periods of incubation. Colony counts of the yeasts were determined after incubations in several concentrations of polymyxin B for the times noted. The MIC was defined as that concentration of polymyxin B which completely prevented cell multiplication (colony counts at the end of the incubation period equal to initial inoculum).* (○) *C. albicans;* (●) *S. cerevisiae.*

DISCUSSION

The two yeasts used in our studies were both susceptible to relatively low concentrations of polymyxin B. The fact that *S. cerevisiae* was more susceptible than *C. albicans* may be due to differences in their cell membranes. Newton has observed the antibacterial activity of polymyxin B to be most potent against gram-negative organisms and has suggested that a difference in membrane phospholipid content accounted for this selectivity (4). Varying susceptibilities within the genus *Candida* have also been explained by a similar mechanism (5). The increase in MIC that we have observed on prolonged incubation may result from selection of resistant organisms, or it may reflect inactivation of the drug over prolonged time periods.

Nicholls was unable to demonstrate any polymyxin B effect in the 45 clinical isolates of *C. albicans* that he examined, whereas each of 12 *C. tropicalis* strains studied was susceptible to the drug (5). In determining susceptibility to polymyxin B, the culture media and techniques must be sufficient to insure optimal growth conditions. In studies with blue-green algae, Whitton showed that polymyxin B was most effective against actively growing, as opposed to older, cell cultures, and postulated that polymyxin B function required a functional, metabolizing cell (8). We preincubated our yeasts overnight prior to use in these investigations, whereas

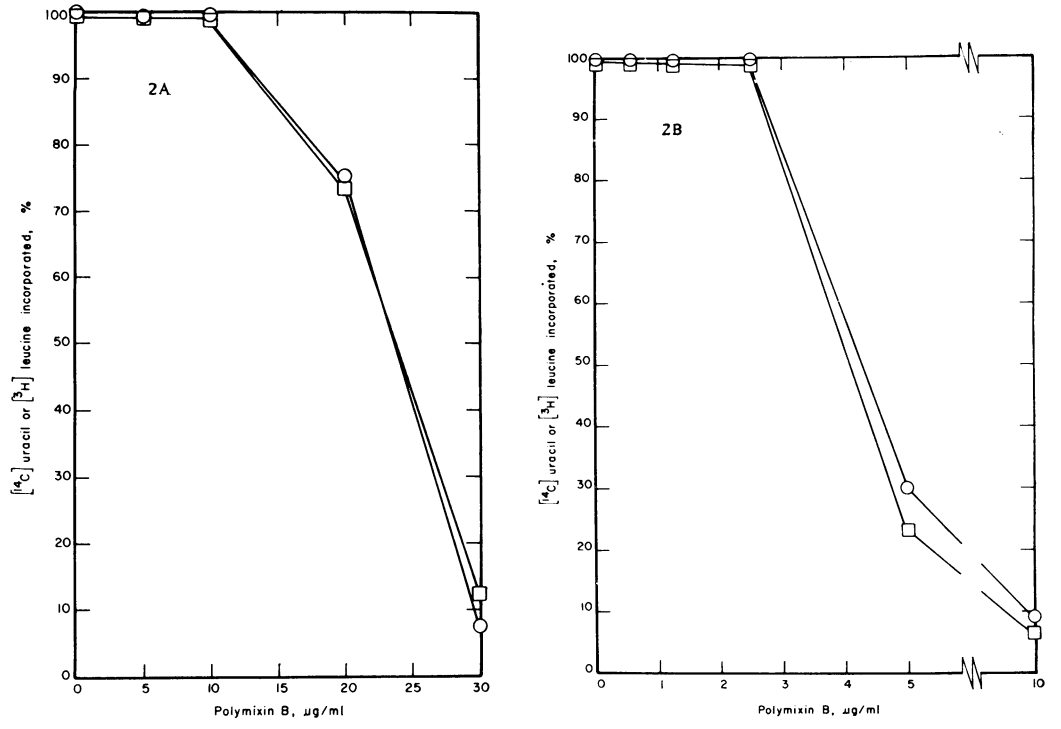

FIG. 2. *Dose response to polymyxin B of* [^{14}C]*-uracil and* [^{3}H]*-leucine incorporation into trichloroacetic acid-precipitable fraction of C. albicans (A) and S. cerevisiae (B). The yeasts (10^{6} cells/ml) were grown in* [^{3}H]*-leucine (0.5 µCi/ml) and* [^{14}C]*-uracil (5 µCi/ml) at the indicated concentrations of polymyxin B for 4 hr. The 100% values for* [^{3}H]*-leucine were 10,000 counts per min per ml, and for* [^{14}C]*-uracil, 1,600 counts per min per ml. (○)* [^{3}H]*-leucine; (□)* [^{14}C]*-uracil.*

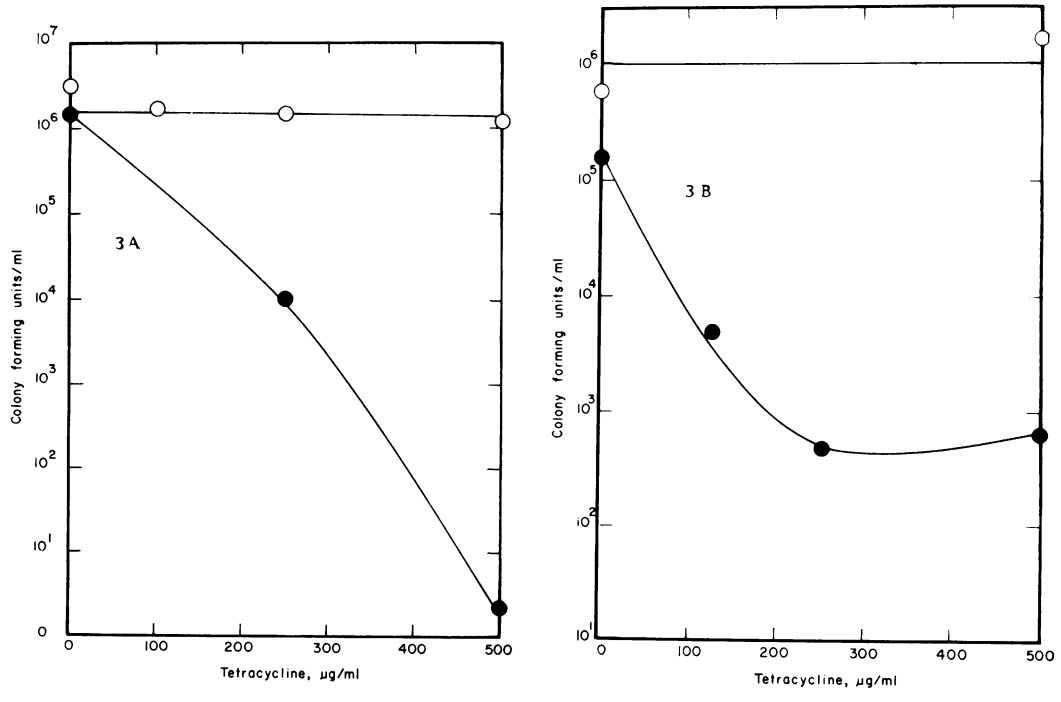

FIG. 3. *Dose-response curve of the viability of C. albicans (A) and S. cerevisiae (B) to tetracycline alone and in combination with polymyxin B. Incubations were for 3 days, and viability studies were performed by colony count determinations. Polymyxin B concentrations were 12.5 µg/ml for C. albicans (A) and 2.5 µg/ml for S. cerevisiae (B). (○) Without polymyxin B; (●) with polymyxin B.*

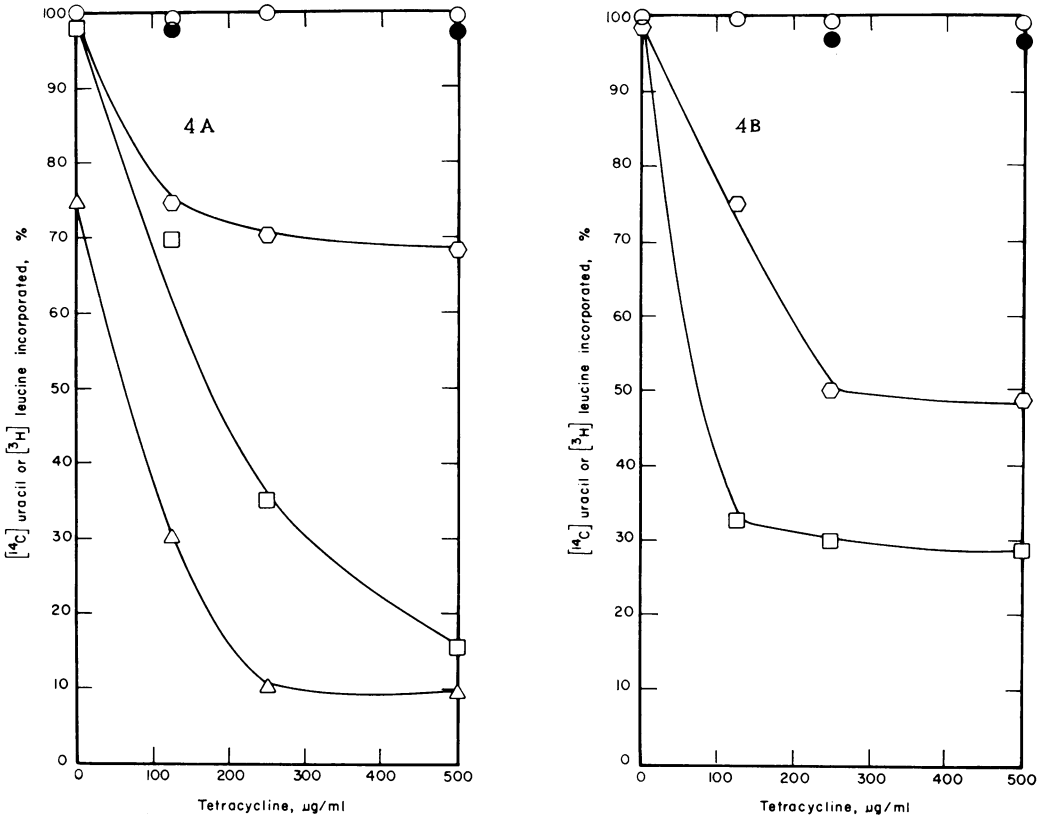

FIG. 4. *Dose response to tetracycline of* [^3H]*-leucine (0.5 µCi/ml) and* [^{14}C]*-uracil (5 µCi/ml) incorporation after 4 hr of incubation of C. albicans (A) and S. cerevisiae (B) in the presence or absence of polymyxin B. The 100% values for* [^3H]*-leucine incorporation in the tubes without polymyxin B were 13,516 counts per min per ml (A), 15,016 counts per min per ml (B); for the polymyxin B-treated cells, 100% values were 14,808 counts per min per ml (A), 14,837 counts per min per ml (B). (A) Polymyxin B concentrations:* (○) *no polymyxin B,* (⬡) *5 µg/ml,* (□) *10 µg/ml,* (△) *20 µg/ml;* (●) [^{14}C]*-uracil incorporation with 5 µg of polymyxin B per ml. (B) Polymyxin B concentrations:* (○) *no polymyxin B,* (⬡) *1.25 µg/ml,* (□) *2.5 µg/ml;* (●) [^{14}C]*-uracil incorporation in 1.25 µg of polymyxin B per ml.*

Nicholls exposed stationary cells to the drug. Possibly some of the discrepancies between our data and his may have resulted from differences in medium or a relative resistance of the slowly metabolizing yeast cells used in his studies. Our studies were done on only one isolate each of *C. albicans* and *S. cerevisiae*. We will have to examine many more strains before judging whether polymyxin B susceptibility is a common characteristic of yeasts.

The strong reduction in viability of the yeasts with the use of the two drugs in combination resulted from the potentiation of tetracycline by polymyxin B. This conclusion is supported by the data which showed that the drug combination specifically affected protein synthesis and not RNA synthesis. If, instead, tetracycline were potentiating polymyxin B, the pattern of cell death would have conformed to that seen for excess polymyxin B (Fig. 2A and B). Studies with polymyxin B on bacteria have confirmed the ability of this agent to break down permeability barriers and allow materials to flow into the cell. *N*-tolyl-2-naphthylamine-8-sulfonic acid (TNS), a fluorescent dye which is detectable only when combined with protein, was nonfluorescent when exposed to polymyxin B or to bacteria. When polymyxin B was added to the bacteria which had been treated with TNS, fluorescence was detected, indicating the uptake of the dye and its binding to proteins within the cell (4).

The antagonism between polymyxin B and tetracycline when added simultaneously is best viewed with regard to the ionic properties of polymyxin B. Polymyxin B has been shown to react with any of a number of acidic substances

(4). Tetracycline HCl, in a 2% aqueous solution, has a *p*H of 2.1 to 2.3, and its interaction with polymyxin B might prevent the latter from attaching to the cell membrane. With pretreatment of the yeasts with polymyxin B, permeability was already affected so that the tetracycline could penetrate through the cell membrane.

The selective alteration, by polymyxin B, of cell membrane permeability and potentiation of a second agent by increased entry into the cell is similar to the amphotericin B effect in yeast which we described earlier (Kwan et al., Bacteriol. Proc., p. 179, 1972). Although the sites of action in the cell membrane of these two agents differ, the principle of increasing cell permeability to other drugs is the same.

The concentrations of both agents needed to achieve synergy make it unlikely that it will have clinical application. However, these studies suggest that alteration of cell permeability to achieve synergy can be attained with agents other than amphotericin B, and selective permeability changes may be achievable with various agents that bind to membrane constituents.

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