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Influence of Antioxidants on the Bioactivity of Amphotericin B

FRED A. ANDREWS,* WILLIAM H. BEGGS, AND GEORGE A. SAROSI

General Medical Research Service, Veterans Administration Hospital, Minneapolis, Minnesota 55417

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Four antioxidants, propyl gallate, butylated hydroxyanisole, butylated hydroxytoluene, and D-α-tocopherol acid succinate were found to stabilize amphotericin B and to prolong its antifungal activity against *Candida albicans*. Although each of the antioxidants was effective in this respect, propyl gallate and butylated hydroxyanisole were better than butylated hydroxytoluene and D-α-tocopherol acid succinate. None of the antioxidants alone adversely affected normal cell growth. It is suggested that amphotericin B instability is due, at least in part, to lability of the carbon-carbon double bonds of the polyene moiety toward autoxidation. By protecting the drug molecule with an antioxidant, it is possible to significantly lower the quantity of AB necessary to obtain particular antifungal effects.

Amphotericin B (AB), in spite of its instability (6, 10, 11, 16, 21) and high toxicity (11, 13, 14, 20), is the most important single antibiotic for treatment of the systemic mycoses (1). Since AB is a polyunsaturated molecule containing seven conjugated carbon-carbon double bonds (11), it is not surprising that the drug is unstable. One would expect the molecule to autoxidize in the presence of air (either under anhydrous or solvated conditions) with the formation of free radicals, hydroperoxides, peroxides, oxiranes, and carbonyl compounds, as is typical of unsaturated lipids (19). Formation of these highly reactive intermediates would not only reduce the effective drug concentration in solution but would also quite likely contribute to its toxicity, as has been shown for a number of unsaturated lipids (2, 8, 9, 12, 15, 18).

If instability of AB involves autoxidation of the conjugated double bonds, the addition of an antioxidant to a yeast culture treated with AB should improve stability and therefore biological activity of the antibiotic. There are isolated reports in which it has been observed that antioxidants can retard decomposition of polyenes (3, 4, 16). However, we are not aware of any published work in which the possibility that antioxidants can improve the biological activity of AB (or other polyenes) has been systematically investigated. In the present study, we have tested the effect of four well-known primary antioxidants—butylated hydroxyanisole (BHA), *n*-propyl gallate (PG), butylated hydroxytoluene (BHT) and D-α-tocopherol acid succinate (VE)—in a *Candida albicans*-AB system.

MATERIALS AND METHODS

Test organism. *C. albicans* ATCC 11651 was purchased from the American Type Culture Collection, Rockville, Md. Stock cultures were maintained on Sabouraud dextrose agar (Difco) and passed at weekly intervals.

Antibiotic. AB in the form of Fungizone was purchased from E. R. Squibb & Sons, Princeton, N.J. This product was supplied as a sterile lyophilized powder containing 50 mg of AB, 41 mg of sodium desoxycholate, and 25.2 mg of sodium phosphates as buffer. Stock solutions of the drug were prepared in sterile deionized water. In each experiment, freshly prepared solutions, protected from light, were used.

Antioxidants. The four primary antioxidants, PG, BHA, BHT, and VE, were purchased from Sigma Chemical Co., St. Louis, Mo. Each compound was dissolved and diluted in dimethyl sulfoxide (Me_2SO). Concentrations of antioxidant stock solutions were such that the volumes of Me_2SO added to cell suspensions were $\leq 1\%$ of the total volume. At a concentration of 1%, Me_2SO alone or in combination with 0.1 μg of sodium desoxycholate per ml had no observable effect on either the growth of *C. albicans* or on the bioactivity of AB.

Culture medium. The synthetic liquid medium used in these studies had the same composition as that described by Shadomy (17) and contained 6.7 g of yeast nitrogen base (Difco), 1.5 g of L-asparagine, and 10.0 g of dextrose in 1 liter of distilled water. For our purposes, however, the medium was adjusted to pH 7 before filter sterilization.

Experimental. *C. albicans* was grown in synthetic liquid medium at 37°C with rotary shaking (150 rpm). Cultures grown for 12 to 16 h were diluted in fresh medium to a calculated optical density (OD) at 420 nm of 0.008, which represents 3×10^5 to 5×10^5 colony-forming units/ml, as determined by standard dilution and plate count techniques.

In the initial screening experiments designed to compare the four antioxidants, 20-ml portions of cell suspension containing either no drug or 0.10 μg of AB per ml were added to 50-ml Erlenmeyer flasks containing various amounts of the specific antioxidants such that the final concentrations ranged from 0 to 8.0 μg/ml. The mixtures of cells, antibiotic, and antioxidant were then incubated for 24 to 29 h at 37°C with shaking in the dark, and OD values were read on a spectrophotometer (Coleman model 6C) at 420 nm. Three control flasks were included in each experiment: flask 1 contained cells only, flask 2 contained cells plus 8.0 μg of antioxidant per ml, and flask 3 contained cells plus 0.10 μg of AB per ml.

In growth experiments designed to show the enhanced effects of antioxidants on the antifungal activity of AB in more detail, three concentrations of AB were used, with the incorporation of a constant ratio of drug to antioxidant. *C. albicans* cultures were diluted as described above, and 75-ml volumes of cell suspension were placed in 500-ml Nephelo-Culture flasks (Bellco) containing sufficient antioxidant to give the desired final concentrations. Drug was then added at 0.025, 0.05, or 0.10 μg of AB per ml. Controls included 75-ml volumes of cell suspension with either no additions, AB alone at the three different concentrations, or antioxidants alone at 0.60 μg/ml. A drug/antioxidant ratio of 1:6 was

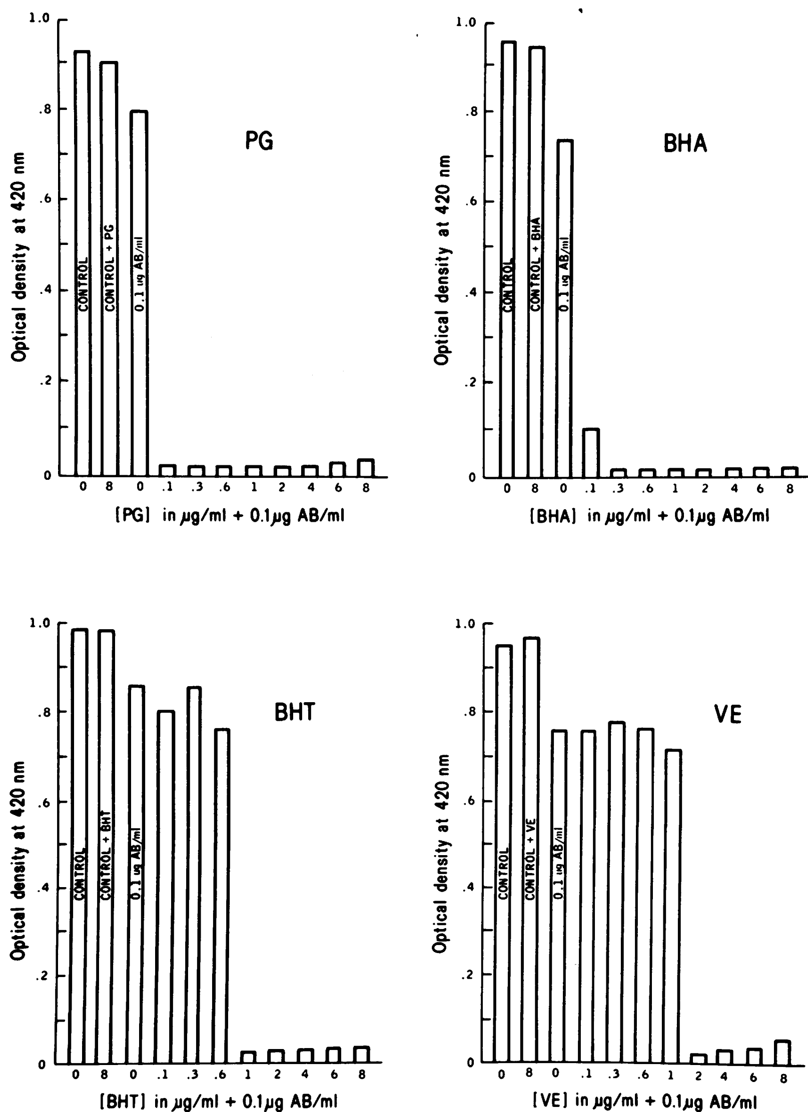

FIG. 1. *Effect of four antioxidants on the bioactivity of AB against C. albicans. Growth was assayed turbidimetrically after 24 to 29 h of incubation at 37°C. The time zero OD was approximately 0.008. Control cultures contained no AB.*

maintained (e.g., 0.10 μg of AB plus 0.60 μg of BHA per ml). The flasks were incubated with shaking at 37°C in the dark and OD values were read at time intervals over a 72-h incubation period.

RESULTS

Growth data shown in Fig. 1 support the contention that primary antioxidants preserve the structural integrity of AB and prolong its biological activity. PG and BHA were highly effective over the entire range of concentrations tested. Growth in cultures treated with 0.10 μg of AB per ml alone approached that of the controls in 24 to 29 h, but when PG or BHA was also present at 0.10 to 8.0 μg/ml, growth remained completely inhibited. BHT did not protect AB from loss of activity below 1.0 μg/ml, and the lowest effective concentration for vitamin E was approximately 2.0 μg/ml. Since each compound has a different oxidation-reduction potential, variations in effectiveness would be expected. None of the antioxidants alone, at a level of 8.0 μg/ml, adversely affected cell growth.

To further demonstrate the improved antifungal effect of AB on *C. albicans* in the presence of an antioxidant, detailed growth experiments were conducted with three different concentrations of AB and a constant ratio of drug to antioxidant (1:6). In these studies, the effects of the two agents alone and in combination were measured turbidimetrically over prolonged incubation periods. Data for the antioxidant BHA are shown in Fig. 2. It can be noted that AB at 0.025, 0.05, and 0.10 μg/ml inhibited growth for varying periods of time that were directly proportional to the initial drug concentrations used. On a microgram basis, AB to BHA ratios of 1:6 approximately doubled the time period that each level of drug completely prevented growth. It should also be noted that growth in the presence of BHA alone at a level of 0.60 μg/ml was the same as in the control culture containing no drug or antioxidant. In identical experiments using PG instead of BHA, the results were even more dramatic as shown in Fig. 3. The combination of 0.05 μg of AB and 0.30 μg of PG per ml of cell suspension appeared to be as effective as 0.10 μg of AB plus 0.60 μg of BHA per ml (Fig. 2), thus indicating the superiority of PG as an antioxidant under the test conditions used. Although it is not shown in Fig. 2 and 3, 0.10 μg of AB plus 0.60 μg of BHA per ml and 0.05 μg of AB plus 0.30 μg of PG per ml inhibited cell growth as well as did 0.20 μg of AB per ml alone.

DISCUSSION

The data presented above and the observations of Rickards et al. (16) leave little doubt that the instability of AB is due, at least in part, to lability of its polyene structure to autoxidation. The drug appears to behave much like an unsaturated lipid in the presence of molecular oxygen in that the molecule is stabilized and its bioactivity is preserved by primary antioxidants.

It is reasonable to assume that peroxidation of AB occurs in a relatively short time in the presence of air, and our data suggest that the intermediates resulting from this process are not biologically active. Since neither the lactone ring nor the other functional groups are

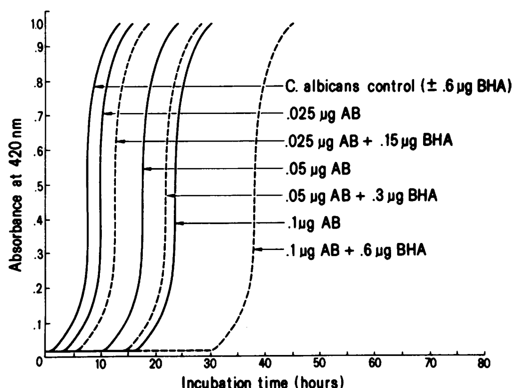

FIG. 2. *Growth inhibition of C. albicans by three different concentrations of AB and the effects of BHA on the duration of inhibition. The time zero absorbance (OD) was approximately 0.008. Growth was measured turbidimetrically and plotted as a function of time. Numbers represent micrograms of AB or BHA per milliliter of cell suspension.*

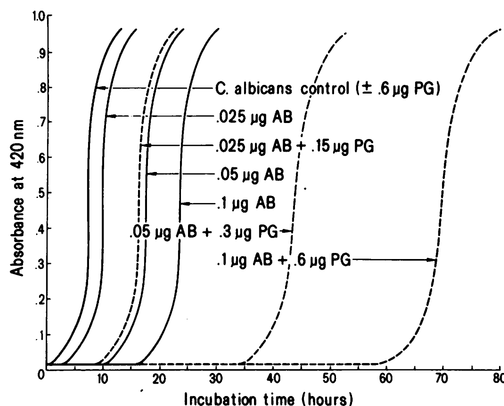

FIG. 3. *Growth inhibition of C. albicans by three different concentrations of AB and the effects of PG on the duration of inhibition. The time zero absorbance (OD) was approximately 0.008. Growth was measured turbidimetrically and plotted as a function of time. Numbers represent micrograms of AB or PG per milliliter of cell suspension.*

affected by small amounts of added antioxidants, it is reasonable to assume that the polyene region of the AB molecule is primarily responsible for instability under rather mild conditions.

PG, BHA, and BHT are widely used as antioxidants in the food and pharmaceutical industries and are generally regarded as safe for human consumption (5, 7). Similarly, VE is nontoxic, but is a less effective antioxidant than the other three considered here. Since these compounds are well tolerated by animals and humans and since they can stabilize and prolong the biological activity of AB, antioxidants may find practical application in AB chemotherapy. It might be possible to design effective treatment regimens on the basis of low-dose AB in combination with an antioxidant, thereby reducing AB toxicity. Although the possibility is purely speculative at this point, an antioxidant might also reduce toxicity by preventing the formation and interaction of highly reactive peroxides with tissue proteins, as is the case with other peroxidized lipids in vivo (8, 18).

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