Selective Membrane Toxicity of the Polyene Antibiotics: Studies on Lecithin Membrane Models (Liposomes)

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In the absence of sterol, amphotericin B at $5 \times 10^{-4}$ M caused maximum marker release from the saturated dipalmitoyl lecithin liposomes, minimum release from the unsaturated dioleoyl lecithin liposomes, and an in-between response from egg lecithin liposomes. Nystatin at 2.5 to $4.0 \times 10^{-4}$ M induced appreciable marker release from all three types of sterol-free liposomes. The amphotericin B- and nystatin-induced permeability changes in dipalmitoyl lecithin liposomes were drastically suppressed by the incorporation of cholesterol or stigmasterol (with identical $\Delta_5$ sterol nuclei), but were unaffected by the incorporation of ergosterol or 5,7-cholestadien-3$\beta$-ol (with identical $\Delta_5$,7 sterol nuclei). The nystatin sensitivity of dioleoyl lecithin liposomes remained low after the incorporation of cholesterol or stigmasterol, but was greatly enhanced by the incorporation of ergosterol or 5,7-cholestadien-3$\beta$-ol. Digitonin, a compound known to interact specifically with membrane sterol, induced marker release from liposomes in proportion to the amount of either cholesterol or ergosterol incorporated; epicholesterol did not sensitize to digitonin. These results lead to the following conclusions: (i) polyene-induced permeability alteration in model membrane systems is effected by the composition of membrane phospholipid fatty acyl chains; (ii) the distribution of double bonds in the sterol nucleus is related to the selective toxicity of the polyenes toward natural sterol-containing membranes; and (iii) polyenes differ in membrane selectivity.

The polyene antibiotics are a group of structurally related macrolides which damage certain biological membranes and are used clinically for their fungicidal activity. The widely held concept on the mode of their antifungal action is that all polyene effects are due to their binding to membrane sterols (4, 9, 12, 22). Research efforts during the past decade have been devoted primarily toward the demonstration of direct interaction between polyene and sterol. These studies have been fruitful (10, 12, 14, 15, 18). In particular, filipin and cholesterol, the most studied polyene-sterol system, were shown to complex in a stoichiometrically and stereochemically defined manner (14, 18).

Two questions pertinent to the antimembrane action of the polyenes have not been systematically studied, namely: (i) what is the possible role of other membrane components, such as phospholipid, in conferring polyene-sensitivity; and (ii) what is the basis for the differential toxicity of polyenes versus membranes containing various sterols? In the present work, the effect on several membrane systems of two clinically useful polyenes, amphotericin B and nystatin, were studied as part of an effort to answer the questions. In this paper, our results with lecithin liposomes are presented; a brief preliminary report of some of these data has appeared (5). In the accompanying paper (6) our studies of selected natural membranes are presented.

Previous studies on polyene-model membrane interaction (10, 14, 15, 18) were centered around the interaction of filipin with cholesterol in systems with egg lecithin as the only phospholipid. It is of interest that although filipin is a neutral polyene, all five polyenes approved for clinical use (amphotericin B, candididin, nystatin, pimaricin, and trichomycin) (17) are amphoteric molecules, and although cholesterol is the major sterol of most mammalian cells, it
has not been detected in yeast membranes in significant amounts (7).

The liposome preparations employed in this study were derived from three different types of lecithin, the saturated dipalmitoyl lecithin, the unsaturated dioleoyl lecithin, and the lecithin mixture isolated from egg yolk which contains saturated and unsaturated fatty acid residues. Sterols structurally related either to cholesterol, the major sterol in most mammalian cells, or to ergosterol, the major sterol in most yeasts (7), were incorporated into these liposomes at various sterol-lecithin molar ratios. The sensitivity of the membranes to the polyene was determined by the release of trapped glucose marker by using a modification of the procedures developed by Kinsky and his co-workers (10).

The results demonstrate that: (i) the response of a liposome membrane system toward polyenes is affected by the apolar aspects of the phospholipid; (ii) the structure of the membrane sterol is an important variable in determining polyene sensitivity, and (iii) polyenes differ in their membrane selectivity.

MATERIALS AND METHODS

Liposomes were prepared by mixing a chloroform solution of lecithin, dicetyl phosphate, and sterol (when indicated) in the desired molar ratio. Dicetyl phosphate was present in all liposome preparations at a lecithin-dicetyl phosphate molar ratio of 1:0.05. To test for polyene or digitonin susceptibility, 0.1 ml of the liposome suspension, at 0.5 μmol of phosphate per ml of saline, was added to tubes containing 0.15 M NaCl, 50 mM tris(hydroxymethyl)aminomethane buffer (pH 7.5), and various amounts of polyene or digitonin in a final volume of 1.0 ml. After incubation at the indicated temperature for the given time intervals, the reaction mixtures were centrifuged at room temperature, and the glucose content of the clear supernatant fluid was quantitated enzymatically. The total amount of trapped glucose was determined after treatment of the liposomes with Triton X-100. The extent of glucose marker release is expressed as "percentage of maximum glucose released," and was calculated from the expression: [(glucose release in the presence of polyene - blank control)/total amount of glucose trapped - blank control] × 100. The details of the procedure were described in a previous report (5).

Stock solutions of amphotericin B (generously supplied by the Squibb Institution for Medical Research, New Brunswick, N.J.), nystatin (purchased from Nutritional Biochemical Corp., Cleveland, Ohio), and digitonin were made in dimethylformamide and stored at −15 C. Dimethylformamide was present to a final concentration of 0.5% (vol/vol) in all the experiments with liposomes. Sterols were purchased from Steraloids Inc. (Pawling, N.Y.). The structures of the various sterols used are depicted in Fig. 1. Egg lecithin was isolated and purified by standard procedures (21). Synthetic dipalmitoyl-L-lecithin was obtained from Calbiochem (San Diego, Calif.), and dioleoyl-L-lecithin was from Supelco, Inc. (Bellefonte, Pa.). These lipids were homogenous as evidenced by yielding single spots by thin-layer chromatography on silica gel G plates developed by using chloroform-methanol-water (65:25:4).

RESULTS

The polyenes available to us were not of uniform purity, and it is difficult to make comparisons on a weight basis. The polyene concentrations chosen for these experiments were based roughly on our earlier liposome studies (5) and on the data presented in the accompanying paper (6), which examines the potency of the antibiotics against natural membranes. Amphotericin B was added at approximately 5.0 × 10⁻⁹ M (~5 μg/ml) and nystatin was employed at a concentration of approximately 2.5 to 4.0 × 10⁻⁴ M (~25-40 μg/ml).

The effect of lecithin fatty acyl chain composition and the influence of incorporation of various sterols on polyene-liposome interaction are compiled in Fig. 2 and 3. In the absence of sterol, amphotericin B induces appreciable glucose leakage from the saturated dipalmitoyl lecithin liposomes. This effect is suppressed by the incorporation of cholesterol or epicholesterol, but is minimally influenced by ergosterol incorporation (Fig. 2a). The sterol-free unsaturated dioleoyl lecithin liposomes are unaffected by amphotericin B; the antibiotic action is potentiated dramatically by the incorporation of either ergosterol or cholesterol, but not by epicholesterol (Fig. 2b). An intermediate response to amphotericin B from the sterol-free egg lecithin liposome system is augmented by the incorporation of ergosterol and, to a lesser

![Fig. 1. Chemical formulas of sterols used in this study.](http://aac.asm.org/...
Inhibiting response of results towards natural terol activity toward natural sterol systems may be caused by cholesterol; epicholesterol incorporation inhibits the damage (Fig. 2c). The inhibition of amphotericin B-induced liposome response in the presence of high molar concentrations of cholesterol (Fig. 2) is of interest and will be discussed subsequently.

Nystatin induced marker release from all three types of sterol-free liposomes in the order egg lecithin > dipalmitoyl lecithin > dioleoyl lecithin (Fig. 3). Although the incorporation of ergosterol leads to an enhanced or unchanged response to nystatin, the incorporation of increasing amounts of cholesterol or epicholesterol results in an almost linear decrease of nystatin sensitivity in all liposomes studied, regardless of their fatty acyl chain composition. The data are consistent with the nystatin selectivity toward natural sterol-containing membrane systems described in the accompanying paper.

These data suggest that the molecular basis for the selective toxicity of the polyenes may be related to the structure of sterols present in natural membranes as well as to the apolar aspects of the phospholipids. The structural requirements for optimum polyene-sterol interaction in a liposome system have been detailed by Norman et al. (14, 15): a planar sterol nucleus, an intact side chain at C-17, and a 3β-hydroxyl group. The critical role of the 3-hydroxyl group configuration is confirmed by our data. Epicholesterol with its 3α-hydroxyl configuration drastically suppresses polyene-induced responses. It is also evident, however, that the two naturally occurring sterols, cholesterol and ergosterol, while meeting all the structural requirements defined above, differ considerably in their impact on polyene susceptibility in some membrane systems. In comparison, digitonin, another compound known to interact specifically with membrane sterols, was shown to induce permeability changes from liposomes in a manner strictly proportional to the amount of 3β-hydroxyl sterol incorporated, regardless of the liposome fatty acyl chain composition (Fig. 4), which suggests that the effect of digitonin is due exclusively to its binding with sterol, regardless of the apolar aspect of the lecithin. The effect of polyene is apparently a far more subtle and complex one. One must take the over-all state of membrane organization into consideration, not just the presence of a single specific membrane component (5).

In view of the results shown in Figs. 2 and 3, additional experiments were initiated to examine systematically the effect of sterol structural variation on polyene-induced membrane permeability alteration. Ergosterol differs structurally from cholesterol in that it has two more double bonds (Δ7 and Δ22) and an additional 24β-methyl group. The effect of the following sterols were also studied in the liposome system in addition to cholesterol and ergosterol: 5,7,7′-cholestadien-3β-ol, which has a nucleus structure identical to ergosterol and C17 side chain structure identical to cholesterol; stigmasterol, which has a nuclear struc-

**Fig. 2.** Effect of epicholesterol (●), ergosterol (■), and cholesterol (▲) incorporation into various lecithin liposomes on the extent of glucose marker release in the presence of 5 μg of amphotericin B per ml. In Fig. 2a the lecithin is dipalmitoyl lecithin, in 2b it is dioleoyl lecithin, and in 2c it is egg lecithin. In all cases, the concentrations of sterol are expressed as molar percent of phospholipid phosphorus. Liposomes with the test agents, as well as controls, were incubated at room temperature for 2 h. Details are described in the text.

**Fig. 3.** Effect of epicholesterol (●), ergosterol (■), and cholesterol (▲) incorporation into various lecithin liposomes on the extent of glucose marker release in the presence of 25 μg of nystatin per ml. The lecithins studied in Fig. 3a, 3b, and 3c are dipalmitoyl, dioleoyl, and egg lecithin, respectively. Experimental conditions were identical to those described for Fig. 2.

**Fig. 4.** Effect of epicholesterol (●), ergosterol (■), and cholesterol (▲) incorporation into various lecithin liposomes on the extent of glucose marker release in the presence of 100 μg of digitonin per ml. Fig. 4a depicts the results with dipalmitoyl lecithin, 4b with dioleoyl lecithin, and 4c with egg lecithin. Experimental conditions were identical to those described for Fig. 2.
ture identical to cholesterol and a C-17 side chain structure similar to ergosterol; 4,6-cholestadien-3β-ol, which differs from 5,7-cholestadien-3β-ol only in the distribution of its two nucleus double bonds; and dihydrocholesterol, which differs from cholesterol in that it contains no double bond.

One complication has to be taken into consideration. It has been shown that different 3β-hydroxysterols are not incorporated into lecithin liposome to the same extent (2). Thus, it is important for these studies to have an internal control which will allow one to follow certain characteristics of liposomes which depend on and are proportional to the incorporation of sterols. Based on laser Raman spectroscopy, Lippert and Petricolas demonstrated that the effect of cholesterol incorporation into dipalmitoyl lecithin results in a change in the sharp, cooperative gel-liquid crystal transition to a diffuse, noncooperative event (13). We have observed that in the dipalmitoyl lecithin liposome system, with a transition temperature of 41°C (1), there is always a drastic increase in the leakage of markers when the temperature is raised from 37°C to just above 41°C. In Fig. 5 leakage of marker is graphically presented as a function of temperature for liposomes containing various amounts of cholesterol; incorporation of cholesterol at 50 molar percent or more abolishes the sharp transition temperature effects, presumably by preventing the cooperative event which leads to the sudden permeability increase.

In Fig. 6, the leakage of marker glucose at 43°C is given as an internal control to monitor the incorporation of different sterols into dipalmitoyl lecithin liposomes. In each instance, the different sterols are incorporated, resulting in decreasing leakage at 43°C with increasing molar percent of sterol incorporated. The release of marker at 37°C in all cases was practically identical to those at 4°C. This approach is impractical for dioleoyl lecithin liposomes, because that lipid has a transition temperature below 0°C. Instead, the effect of digitonin is used as the internal control in the experiments with dioleoyl lecithin (Fig. 7), because the disruption by digitonin depends on the incorporation of 3β-hydroxy sterol; with stigmasterol and ergosterol near maximum digitonin susceptibility occurs at 15 molar percent. Once it became apparent that the composition of lecithin fatty acyl chain plays a central role in polyene susceptibility, it became important to study only liposomes prepared from defined lecithins. Egg lecithin, like most other natural glycerophospholipids, is a mixture with both saturated and unsaturated fatty acyl chains (11); it was not further studied.

As observed before, dipalmitoyl lecithin liposomes respond almost identically toward both amphotericin B and nystatin (Fig. 6). The polyene-induced response in this system is: (i) drastically suppressed by the incorporation of stigmasterol, dihydrocholesterol, and cholesterol; (ii) unchanged by the incorporation of 5,7-cholestadien-3β-ol and ergosterol; and (iii) moderately suppressed by the incorporation of 4,6-cholestadien-3β-ol (Fig. 6).

The results with liposomes derived from dioleoyl lecithin are presented in Fig. 7. Although amphotericin B-susceptibility is augmented by the incorporation of all sterols under study, the effect of incorporation of the various sterols on nystatin susceptibility can be categorized in a way similar to that described with dipalmitoyl lecithin liposome systems. Stigmasterol, dihydrocholesterol, and cholesterol have no effect on nystatin susceptibility. 5,7-Cholestadien-3β-ol and ergosterol markedly augment nystatin susceptibility: 4,6-cholestadien-3β-ol falls in between the others.

It is evident that the difference between ergosterol and cholesterol in conferring polyene sensitivity derives from the distribution of dou-

![Fig. 5. Effect of temperature on the extent of glucose marker released from dipalmitoyl lecithin liposomes containing cholesterol at: 0 (C), 25 (●), 50 (▲), and 75 (■) molar percent (relative to lecithin). The liposomes were incubated at the indicated temperature for 1 h. The percent of maximum glucose release is calculated from the expression: % maximum glucose release = ([glucose release at given temperature / glucose release at 4°C] - [glucose release at 4°C]) x 100.](http://aac.asm.org/)
ble bond in the sterol nucleus and that the presence of a double bond in the C-17 side chain plays a less crucial role.

**DISCUSSION**

The results demonstrate that: (i) the response of a liposome model membrane system toward polyenes is affected by its fatty acyl chain composition (Fig. 2 and 3), (ii) incorporation of cholesterol suppresses nystatin sensitivity in all three types of liposomes tested (Fig. 3); (iii) ergosterol shows a far more general effect by conferring sensitivity toward both amphotericin B and nystatin (Fig. 2 and 3); and (iv) the incorporation of 5,7-cholestadien-3β-ol (with a nucleus structurally identical to ergosterol) and stigmasterol (with a nucleus structurally identical to cholesterol) gave effects similar to ergosterol and cholesterol, respectively (Fig. 6 and 7). These data have significant implications for the role of the sterol nucleus double-bond distribution in the selective toxicity of polyenes toward natural sterol-containing membranes. Confirmation of this has been obtained by our studies of the structural requirement of sterols for conferring polyene sensitivity on membranes of viable cells such as *Acholeplasma laidlawii* B (6).

The relevance of fatty acyl composition of phospholipids in membrane systems has been emphasized before, mainly in connection with their role in fulfilling the fluidity requirement of membrane-related events (3, 8, 16, 24). The effect of fatty acyl chain composition studied and discussed in this report is of a different nature. At room temperature, in the absence of sterol, liposomes derived from dioleoyl lecithin, which have a gel-liquid crystal phase transition temperature well below 0°C, are far less sensitive to polyenes than are liposomes derived from the saturated dipalmitoyl lecithin, which has a transition temperature of 41°C (Fig. 2 and 3). Also, the polyene insensitivity of liposomes containing dipalmitoyl lecithin to cholesterol at a molar ratio of 1:0.5 remains unchanged in the temperature range between 4 and 45°C (5).

It should be pointed out that dipalmitoyl and dioleoyl lecithins have an unnatural composition, and caution may be indicated in attaching too much physiological significance to results obtained with liposomes containing these synthetic compounds.

Polyene-induced permeability alteration in cholesterol-egg lecithin liposomes has been well studied (10, 19, 20, 23) and recently reviewed (9). In studies performed by Weissman and Sessa (19, 20, 23) cholesterol was incorporated to a maximum concentration of 10 molar percent. In studies reported by Kinsky et al. (10), the extent of filipin-induced liposome marker release was proportional to cholesterol incorporation only to 12.5 molar percent; further in-
crease of cholesterol concentration to 33 molar percent resulted in liposomes with less sensitivity toward filipin. Our observations confirm this finding with amphotericin B (Fig. 2c). It is unlikely that such a decline in sensitivity is due to the competition of unincorporated or released cholesterol with intact liposomes for the available antibiotics as suggested by Kinsky et al. (10). Cholesterol is known to be incorporated into egg lecithin liposome up to a molar ratio of approximately 1 (2). Also, in our experience, sterols which are not incorporated into the lecithin liposomes usually crystallize in the presence of aqueous glucose marker and are retained by the Sephadex G-75 column used for removing the untrapped glucose. If the presence of cholesterol were the dominant requisite for polyene susceptibility in membrane systems, one would expect that an increase in polyene-induced permeability would be directly proportional to the molar percent of cholesterol incorporated. In cholesterol-egg lecithin liposome systems, this has not been demonstrated to be the case, whereas digitonin, another compound known to interact specifically with membrane sterols, was shown to induce permeability changes in proportion to the amount of cholesterol incorporated (Fig. 4).

Norman et al. (14, 15) have studied the structural requirement of sterol for optimum polyene interaction and demonstrated the necessity of a planar sterol nucleus, an intact side chain at C-17, and a 3β-hydroxyl group. They also indicated that the introduction of a Δ7 double bond decreased the interaction, that the introduction of a Δ22 double bond enhanced the interaction (14), and that cholesterol followed by dihydrocholesterol and stigmasterol were the most effectively interacting sterols (15). Our data confirm the requirement for a 3β-hydroxyl group. Epicholesterol, with its 3α-hydroxyl configuration, suppressed any polyene-induced response (Fig. 2 and 3). However, our data (Fig. 6 and 7) clearly indicate that the introduction of a Δ7 double bond (ergosterol and 5, 7, 8, 9, 10-pentacholen-3β-ol) greatly enhances the interaction, that the introduction of a Δ22 double bond (stigmasteryl) does not increase the interaction, and that ergosterol and 5, 7, 8, 9, 10-pentacholen-3β-ol, followed by 4, 6-dihydroxyl-3β-ol, are the most effective sterols in sensitizing to liposome damage. This seeming discrepancy is likely based on differences in experimental approaches. Norman et al. (14, 15) emphasized the binding between polyene and sterols, and they also studied the interaction of polyenes with liposomally bound sterols by measuring sterol-induced spectral alterations of the polyenes. Our concern is the polyene-induced membrane permeability alteration; we studied the interaction of polyene with sterol-containing liposomes through the measurement of polyene-induced marker release. The conclusions derived from these differences point to the possibility that the connection between the binding of polyenes by membrane sterols and the polyene-induced membrane permeability alterations are not as direct as previous studies suggested (4, 9, 12, 22). In a previous communication (5), we called attention to the likelihood that the polyene susceptibility of membrane systems relates to the overall state of membrane organization rather than to the binding with a single membrane component such as sterols. The major sterol influence on polyene susceptibility seems to be its effect on membrane organization. The more comprehensive data presented here are consistent with this contention.

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