POLYENE ANTIBIOTIC ACTION ON LECITHIN LIPOSOMES: EFFECT OF CHOLESTEROL AND FATTY ACYL CHAINS

Chuen-Chin HsuChen and David S. Feingold

Departments of Medicine, Beth Israel Hospital

and Harvard Medical School

Boston, Massachusetts 02215

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Summary:

The effect of cholesterol incorporation upon amphotericin B and nystatin susceptibility of lecithin liposome systems containing various fatty acids has been studied. Cholesterol was shown to: 1) confer sensitivity to low concentrations of amphotericin B in liposomes derived from egg lecithin, and 2) suppress the amphotericin B and nystatin-induced response in liposomes derived from dipalmitoyl or distearoyl lecithins. This clear cut difference cannot be explained by mechanisms of drug action so far presented. They are discussed in connection with the possibility that susceptibility to these polyene antibiotics is related to the over-all state of the membrane organization, in particular to the over-all conformation of membrane components.

Introduction:

The polyene antibiotics are remarkable for potent antifungal activity and lack of effect on organisms devoid of membrane sterols. Studies have consistently demonstrated that all of the polyenes interact with the membranes of susceptible cells to alter essential membrane permeability characteristics. The identification of the composition and structure of membranes required for polyene susceptibility is more controversial (1). It is generally accepted, however, that, at low concentrations, all the polyene effects depend upon an initial interaction between the polyene and sterols in the membrane (1,2).

Evidence for the central role of sterols is compelling. 1) Mycoplasma laidlawii were susceptible to filipin (3) or amphotericin B (4) when cultured in the presence of cholesterol; and the susceptible cells were rendered resistant by transfer from cholesterol-containing medium to cholesterol-free medium; 2) the incorporation of cholesterol into egg lecithin liposomes increased their sensitivity to filipin (5), as indicated by trapped marker released.

In this communication, using the same model membrane system described by Kinsky et al (5), we report that cholesterol incorporation into lecithin liposomes

can either augment or suppress the polyene-induced permeability alteration depending on the fatty acid composition of the phopholipid.

Materials and Methods:

Liposomes were prepared by mixing chloroform solutions of lecithin, dicetyl phosphate and cholesterol (when indicated) in the desired molar ratio. The molar ratio of lecithin:dicetyl phosphate was 1:0.05. The solvent was removed under reduced pressure and the dried film was dispersed in 0.3M glucose (pre-heated to 70°C). After 2 hours equilibration at room temperature, the untrapped glucose was removed by passing the liposome suspension through a Sephadex G-75 column and eluting with saline (0.15M NaCl). The final concentration of the liposome solution was 0.5 μmoles P/ml of saline. 0.1 ml of this suspension was added to tubes containing 0.15M NaCl, 50mM Tris buffer (pH 7.5) and various amount of polyene or digitonin in a final volume of 1 ml. After incubating at the indicated temperature for 2 hours, the reaction mixtures were centrifuged at 9,000 x g for 15 minutes (4°C), and the glucose content of the clear supernatant was quantitated enzymatically (5).

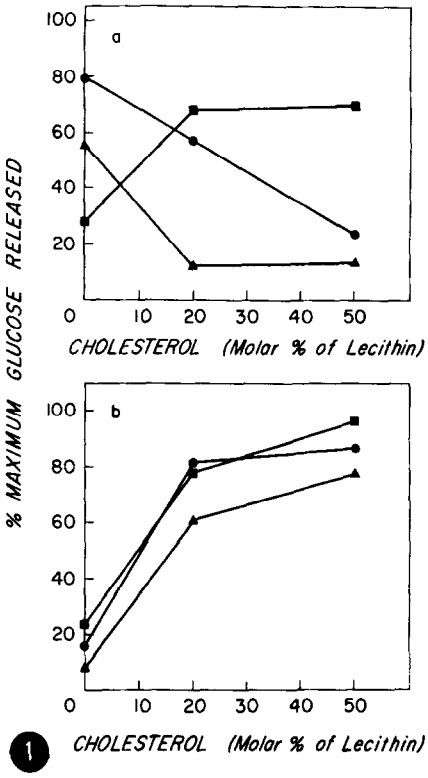
Stock solutions of amphotericin B (Squibb Institute for Medical Research, New Brunswick, N.J.), nystatin (Nutritional Biochemical Corporation, Cleveland, Ohio) and digitonin were made in dimethylformamide and stored at -15°C. Dimethylformamide was present in all samples (including the blank control) to a final concentration of 0.5% (v/v).

In this report, the extent of polyene or digitonin-induced release of glucose marker is expressed as "percent maximum glucose release", and was calculated from the expression:

$$\frac{\text{glucose release in the presence of polyene - blank control}}{\text{total amount of glucose trapped - blank control}} \times 100$$

The total amount of glucose trapped was determined by adding 0.1 ml of liposome preparation to 0.1 ml of 10% Triton X-100, heating in a boiling water bath for a few seconds, and cooling to room temperature prior to glucose assay. The blank controls which did not contain the polyene or digitonin were incubated, centrifuged, and assayed in parallel with the testing samples. The glucose trapping was uniformly excellent with the liposomes examined. The blank controls never exceeded 30% of the total glucose trapped; the blanks were regularly lower with the saturated lecithins, about 10% of the total glucose.

Egg lecithin was isolated and purified by standard procedures (6). Synthetic dipalmitoyl-L-lecithin and distearoyl-L-lecithin were obtained from Calbiochem



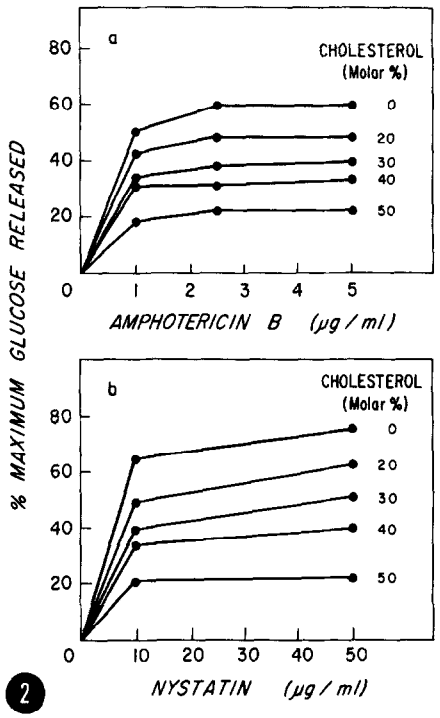


Figure 1. Effect of cholesterol incorporation into liposomes of various lecithins on the extent of glucose marker released by amphotericin B at 5 μg/ml (a) and digitonin at 100 μg/ml (b). Liposomes with the test agents, as well as controls were incubated at room temperature for 2 hours. Details are described in the text. The results with egg lecithin are represented by the squares (■); of dipalmitoyl-L-lecithin by the circles (●); of distearoyl-L-lecithin by the triangles (▲).

Figure 2. Effect of polyene concentration on the extent of glucose marker released from dipalmitoyl lecithin liposomes containing different amounts of cholesterol. Experimental conditions were identical to those described for figure 1.

(San Diego, California). These lipids were chromatographically homogeneous on silica gel G thin-layer plates developed in chloroform:methanol:water (65:25:4, v/v/v).

Results:

The influence of the fatty acid components of lecithin on the measured effect of amphotericin B and digitonin is given in figure 1. Liposomes of egg lecithin responded as expected; sensitivity toward amphotericin B was greatly augmented by cholesterol. The presence of cholesterol in liposomes derived from dipalmitoyl lecithin or distearoyl lecithin suppressed their susceptibility toward amphotericin

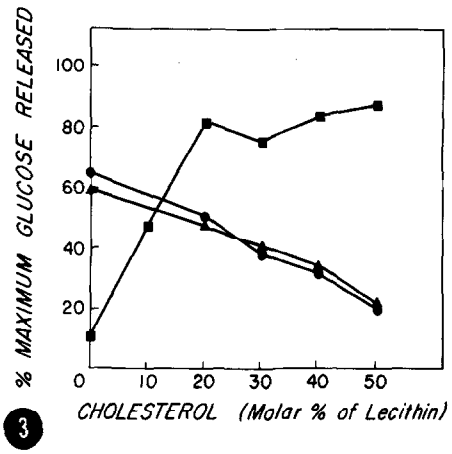
B (figure 1a). These data indicate that amphotericin B can disrupt certain model membranes lacking sterol at concentrations as low as 5×10^{-6}M (about 1 molecule of anitbiotic per 10 molecules of phospholipid in this system). As an internal control the same liposome preparations were incubated under identical experimental conditions in the presence of digitonin, which is known to interact specifically with membrane sterol. Cholesterol was required to confer digitonin sensitivity with all three types of liposomes tested (figure 1b). The egg lecithin liposomes prepared without sterol showed similar low levels of glucose release with both amphotericin B and digitonin, suggesting an absolute sterol requirement for amphotericin B susceptibility with these liposomes.

The effect of cholesterol incorporation on liposomes derived from the lecithin with only the saturated acyl chains was further examined. Figure 2 shows that liposomes of dipalmitoyl lecithin prepared with increasing amounts of cholesterol responded decreasingly toward amphotericin B and nystatin over the concentration range studied. The effect of cholesterol was more evident when the susceptibility of saturated chain liposomes to amphotericin B and nystatin was plotted as a function of the molar percent of cholesterol incorporated and compared with the effect of digitonin (figure 3).

Phospholipid systems are known to exhibit a large variety of organizational forms depending upon the chemical composition, the water content of the system and the temperature. In the fully hydrated state, such as we are studying, the characteristic temperature for gel to liquid crystalline phase transition are ~15 to -5°C for egg lecithin, 41°C for dipalmitoyl lecithin and 58°C for distearoyl lecithin (7). The observed different polyene responses in lecithins with different fatty acid composition could be related in some way to the alterations in phase transition temperature. The polyene sensitivity of liposome prepared from dipalmitoyl lecithin/cholesterol/dicetyl phosphate (1:0.5:0.05, molar ratio) was examined over the temperature range 4 to 45°C (figure 4). Although the action of digitonin was shown clearly to be temperature dependent, the effect of cholesterol on polyene-sensitivity was constant over the range of temperature studied. Preliminary experiments on the polyene susceptibility of dipalmitoyl lecithin liposomes without cholesterol as a function of temperature were performed. These results could not be accurately interpreted because of the drastic increase in glucose released by the blank controls as one approached the transition temperature of 41°C.

Discussion:

Polyenes are known to produce lethal permeability alterations in sensitive cells.



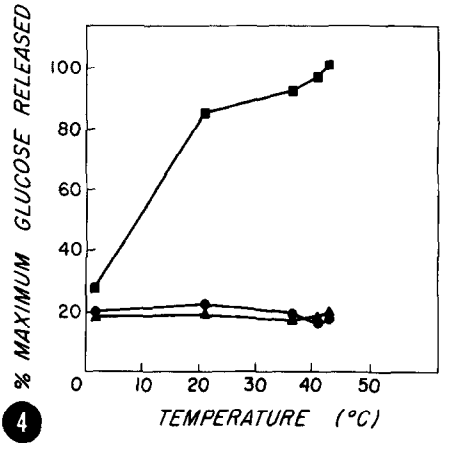


Figure 3. Effect of cholesterol incorporation into dipalmitoyl lecithin liposomes on the extent of glucose marker release in the presence of 100 μg/ml digitonin (■), 5 μg/ml amphotericin B (●) and 10 μg/ml nystatin (▲). Experimental conditions were identical to those described for figure 1.

Figure 4. Effect of temperature on the extent of glucose marker released in the presence of 5 μg/ml amphotericin B (●), 50 μg/ml nystatin (▲) and 100 μg/ml of digitonin (■). Liposome were prepared from dipalmitoyl lecithin/cholesterol/dicetyl phosphate (1:0.5:0.05, molar ratio). Samples were incubated at the indicated temperatures for 2 hours. Experimental details are described in the text.

Most data are compatible with the suggestion that the effect of polyenes depends on the binding to membrane sterols (1,2). Furthermore, direct evidences for interaction of several polyenes and sterols were provided by spectrophotometric studies from different laboratories (8,9,10). These data clearly demonstrate that polyenes interact with sterols, including membrane sterols. However, direct application of such spectrophotometric data to the actual mechanism of the polyene effect on membranes must take the following data into consideration: 1) filipin III, the principal component of the filipin complex, had no effect on the kinetic and equilibrium permeability behavior of water in egg lecithin-cholesterol liposomes although uv absorption and fluorescence polarization studies indicated that filipin III was bound to the liposomes (11); 2) the observation reported here that both amphotericin B and nystatin, at concentrations of 10^{-5} to 10^{-6}M, induce permeability alteration in certain liposomes prepared in the absence of sterol (figure 2a).

Without coming to grips with the sticky proposition that various polyenes may have different modes of action, the effects of cholesterol incorporation studied in this report may be explained by viewing the over-all state of membrane organization in particular the conformation of membrane components, as the major determinant of polyene susceptibility.

Under the conditions of the experiments summarized in figures 1,2, and 3 (room temperature, 22-23°C), dipalmitoyl lecithin and egg lecithin, in the absence of cholesterol, are in gel and liquid crystalline phases, respectively. Both would be in an "intermediate fluid condition" (12) in the presence of 33 mole % of cholesterol. Research reported in the past 2 years makes it possible to define these phase conditions in more precise molecular term (13,14,15,16). At gel phase the hydrocarbon chains of dipalmitoyl lecithin are in a highly ordered all-trans conformation. The presence of unsaturated fatty acid chains as in egg lecithin increases the probability of gauche conformation and results in a more disordered state. The term "intermediate fluid condition" means that hydrocarbon chains in the presence of cholesterol are in a state of conformational restriction intermediate to those in the gel and liquid crystal phases (14). The effects of cholesterol incorporation result in a more ordered egg lecithin by inhibiting formation of some gauche isomer (13,17), and results in a less ordered dipalmitoyl lecithin by reducing the interaction between adjacent hydrocarbon chains (18).

With these theoretical interpretations in mind, the divergent effects of cholesterol on the different membrane models may be resolved by suggesting that a disruptive interaction between polyenes and membranes demands that the membrane be in an ordered state. The extent of this order-requirement may vary among the polyenes, depending on the detailed structure of the individual polyene molecules. Thus, cholesterol may confer amphotericin B and nystatin sensitivity on egg lecithin liposomes by confining the system to a more ordered state. The cholesterol-induced suppression of the polyene sensitivity in dipalmitoyl lecithin liposomes may depend on interruption of the highly ordered conformation of their hydrocarbon chains.

Filipin complex was not used in these studies because of the low potency and hence presumed poor purity of the preparation available to us. A comprehensive study of the action of the various polyenes on several model and biologic membranes is in progress.

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